A potent series targeting the malarial cGMP-dependent protein kinase clears infection and blocks transmission

David A. Baker 1, Lindsay B. Stewart 1, Jonathan M. Large 2, Paul W. Bowyer 1, Keith H. Ansell 2, María B. Jiménez-Díaz 3, Majida El Bakkouri 4,5, Kristian Birchall 2, Koen J. Dechering 6, Nathalie S. Bouloc 2, Peter J. Coombs 2, David Whalley 2, Denise J. Harding 2, Ela Smiljanic-Hurley 2, Mary C. Wheldon 2, Eloise M. Walker 1, Johannes T. Dessens 1, María José Lafuente 3, Laura M. Sanz 3, Francisco-Javier Gamó 3, Santiago B. Ferrer 3, Raymond Hui 4,5, Teun Bousema 7, Iñigo Angulo-Barturén 3, Andy T. Merritt 2, Simon L. Croft 1, Winston E. Gutteridge 1, Catherine A. Kettleborough 2 & Simon A. Osborne 2

To combat drug resistance, new chemical entities are urgently required for use in next generation anti-malarial combinations. We report here the results of a medicinal chemistry programme focused on an imidazopyridine series targeting the Plasmodium falciparum cyclic GMP-dependent protein kinase (PfPKG). The most potent compound (ML10) has an IC 50 of 160 pM in a PfPKG kinase assay and inhibits P. falciparum blood stage proliferation in vitro with an EC 50 of 2.1 nM. Oral dosing renders blood stage parasitaemia undetectable in vivo using a P. falciparum SCID mouse model. The series targets both merozoite egress and erythrocyte invasion, but crucially, also blocks transmission of mature P. falciparum gametocytes to Anopheles stephensi mosquitoes. A co-crystal structure of PvPKG bound to ML10, reveals intimate molecular contacts that explain the high levels of potency and selectivity we have measured. The properties of this series warrant consideration for further development to produce an antimalarial drug.
Malaria, caused by species of the protozoan Plasmodium, remains the most serious parasitic disease in humans. Around 212 million cases of malaria and approximately 429,000 deaths occurred in 2015. Prolonged parasite clearance times, and increasing frequencies of treatment failures following treatment of malaria with artemisinin combination therapies are now regularly reported in parts of Southeast Asia. This has led to concerns about the emergence and spread of resistance to these relatively new medicines, which are the mainstay globally for the treatment of malaria caused by Plasmodium falciparum.

PKG is a cyclic GMP (cGMP)-activated serine/threonine protein kinase that regulates numerous functions in diverse organisms. There is a single PKG gene in malaria parasites and the P. falciparum enzyme, encoded by PfPKG (PlasmoDB Gene ID PF3D7_1436600), has previously been shown to have properties distinct from human orthologues. Selective inhibitors of PKG from the related parasite Eimeria, are thought to interact with a small hydrophobic pocket adjoining the ATP-binding site. Access to this pocket is possible due to the presence of a small (threo-nine) gatekeeper residue in PKG from both Eimeria and Plasmodium (T618 in PfPKG). All mammalian PKGs and most serine/threonine kinases have a large gatekeeper residue preventing access to the pocket and making them insensitive to the PKG inhibitors mentioned above and likely explaining the high levels of selectivity observed.

These PKG inhibitors also block the development of a number of Plasmodium life cycle stages and we have used a chemical genetic approach that exploits the small gatekeeper residue in PfPKG to generate an inhibitor-resistant P. falciparum transgenic line (T618Q), to demonstrate that this enzyme plays an essential role in blood stage replication in the human host as well as gametogenesis and ookinete motility in the mosquito vector. The compounds block P. falciparum blood stage replication by preventing not only schizont rupture and merozoite egress, but also merozoite invasion of red blood stage replication by preventing not only schizont rupture and merozoite egress, but also merozoite invasion of red blood stage replication by preventing rupture of the infected erythrocyte. Inhibition of PfPKG activity also blocks calcium mobilization that is required for merozoite egress and invasion and which is thought to be mediated through phosphoinositide metabolism.

Using a phosphoproteome analysis, we recently identified ~70 P. falciparum proteins expressed in mature schizonts that are phosphorylated in a PKG-dependent manner. These proteins are involved in a wide range of cellular processes including cell signaling, ion/protein transport, chromatin remodeling, and actomyosin motor function.

Here, we show that our newly synthesized imidazopyridine PKG inhibitors have high potency and selectivity against P. falciparum blood stage proliferation in vitro and in vivo, and that they block transmission of gametocytes to Anopheles mosquitoes. We also present co-crystal structures of P. vivax PKG with the inhibitors, which reveal the interactions underpinning the high degree of selectivity we have observed.

**Results**

**Highly potent PKG inhibitors block blood stage proliferation.** Using the Merck imidazopyridine, compound 2 (ML1) (4-[[dimethylamino)methyl]-2-(4-fluoroophenyl)imidazo[1,2-a]pyridine-3-yl)pyrimidin-2-amine), developed to treat Eimeria infection as the chemistry starting point, we synthesized new analogs. These were first tested for their ability to inhibit the kinase activity of recombinant PPKG using a microfluidic mobility shift assay (see Methods). The best IC50 values obtained were <200 pM (Table 1). Selected compounds were also tested in the kinase assay against a recombinant PPKG mutant harbouring a T618Q substitution to investigate the importance of the gatekeeper pocket in the inhibitory mechanism. This mutant kinase exhibited a reduced sensitivity to many of the compounds of between 500 and >100,000-fold (Table 1 and Supplementary Fig. 1), emphasizing that this rare structural feature of PPKG, is vital for inhibitory activity.

The ability of the compounds to block P. falciparum asexual blood stage growth in vitro was then determined using a growth inhibition assay. Compounds had EC50 values ranging from ~500 nM down to <5 nM (Table 1). Selected compounds were then further assayed against the P. falciparum PKG gatekeeper mutant line (T618Q) to determine whether PPKG is their primary target in the asexual blood stages. This line showed between a 10 to >1100-fold reduction in sensitivity to most compounds (Table 1 and Supplementary Fig. 2) confirming on-target activity. Control drugs were tested in parallel to confirm the changed sensitivity of the gatekeeper mutant line was specific. Chloroquine and artemisinin showed equivalent levels of inhibition of both parasite lines, whereas the gatekeeper mutant line is pyrimethamine resistant (as expected) due to the presence of the drug selectable marker included in the plasmid construct used to mediate allelic replacement (Supplementary Table 1). The physical properties of compounds including stability in mouse and human liver microsomes, membrane permeability and lipophilicity were also measured (Supplementary Table 2; Methods) to inform the chemistry programme.

Starting with ML1, and working through several cycles of design and synthesis (see Methods), we generated compounds ML2-10 (Supplementary Fig. 3), which were selected for further analysis (Table 1). ML10 is the most potent with an IC50 of ~160 pM against recombinant PPKG and an EC50 value of ~2 nM against P. falciparum blood stage parasite growth. The compound also exhibited the largest difference in sensitivity between the T618Q transgenic line and the WT 3D7 parasites (>1100-fold). This demonstrates an extremely high degree of specificity for PPKG and indicates that any secondary parasite target is inhibited only at high concentrations of the compound.

**High in vivo efficacy was obtained using the P. chabaudi model.** Compounds ML1, ML4 and ML10 were tested for in vivo efficacy in BALB/c mice infected with P. berghei using a P4-day test at a double daily dose of 25 mg/kg by oral gavage. Reductions in blood stage parasitaemia measured after 4 days’ treatment compared to untreated controls were between 52.0 and 60.4% (Fig. 1a). Plasma samples were taken from satellite groups and the predicted onset of schizogony, led to a 67.9 and 56.5% reduction in parasitaemia, respectively (Fig. 1b). We next compared single and twice daily 50 mg/kg oral doses of ML10. The twice daily dose (3 h apart) aimed to target the entire period of schizont rupture and invasion with the intention of preventing
any reinfection. The single dose experiment achieved a 60.9% reduction in blood stage parasitaemia detected in blood films following 4 days’ treatment, whereas the double dose regimen achieved a mean 97.8% reduction (Fig. 1c). Clearly, the second dose was required to cover the entire period of schizont rupture and re-invasion. There was no overt toxicity in either model. Plasma samples were taken from satellite groups of mice given an oral dose of 25 mg/kg or 50 mg/kg ML10. Supplementary Figure 4b shows a comparison of the mean plasma levels of ML10 measured at both doses. An almost threefold higher concentration (10.4 μM) was measured at the 30-min time point with the higher dose which was still around (8.4 μM) at 60 min. Although this fell to less than 84 nM after 4 h, levels were still well above the EC90 value obtained in the P. falciparum growth inhibition assay. The half-life of ML10 in these experiments was 0.8 h (25 mg/kg) and 1.1 h (50 mg/kg).

**Blood stage infection was cleared in a P. falciparum mouse model.** To further evaluate the efficacy of ML10 it was tested in the GSK P. falciparum mouse model (GSKPfalHuMouse) engrafted with human red blood cells22 by administering twice daily doses of 50 or 100 mg/kg via the oral route to two immunodeficient mice infected with P. falciparum 3D70087/N9 for 4 days. Both doses dramatically reduced parasitaemia (Fig. 2a), with levels of ML10 in plasma far above the EC90 in vitro during the 12-h period monitored (Supplementary Fig. 5). With the higher dose, parasitaemia was not detectable at the end of the treatment. The rate of parasite elimination was at least as good as that obtained with mefloquine. The best estimate of potency was AUCED90 <6.17 μg/ml h, with levels of compound orders of magnitude higher than the EC90 in vitro. After one parasite cycle of exposure, most remaining parasites in peripheral blood were at the late schizont stage (Fig. 2b), suggesting that the process of release of merozoites and invasion of erythrocytes are critical steps of the P. falciparum cycle targeted by ML10. The improved efficacy in the GSKPfalHuMouse model is probably not due to sequence differences between PiPKG and the rodent malaria parasite PKGs, because we previously showed that a transgenic P. berghei line in which Pb PKG was replaced by PfPKG showed equivalent sensitivity to the chemistry start point (ML1)23. It is likely that the higher dose regimen and the resulting higher plasma concentrations of compounds over time obtained in the SCID experiment were responsible for the significant activity observed. The half-life values for ML10 in these experiments was 8.7 h (50 mg/kg) and 17 h (100 mg/kg). Importantly, the blood stage of the P. falciparum life cycle is 48 h compared to 24 h with the rodent malaria parasite species, which likely increases exposure of the sensitive stages to the compound and contributes to the increased efficacy. To investigate the dynamics of parasite killing, an in vitro parasite reduction rate (PRR) assay was carried out24. A lag of about 24 h was observed, during which time the effects of the compounds were reversible following wash-out. However, rapid killing occurred after parasites had been exposed to ML10 for more than 24 h (Fig. 2c). This killing profile is consistent with PiPKG inhibitors acting at the egress/invasion stage as the starting parasite population in this assay is >80% rings. Results are also indicative that inhibition of these cellular processes is lethal and parasites cannot recover even if compound is no longer present. Figure 2c shows the profile of ML10 compared to those of control asexual malarial compounds assayed in the same way.

**The series blocks transmission of P. falciparum to mosquitoes.** We also tested the transmission-blocking effects of this series by pre-incubating mature P. falciparum gametocytes (strain NF54) for 24 h with ML1, ML4, and ML10 and then feeding them to Anopheles stephensi mosquitoes using standard membrane feeding assays (SMFA,25 to measure oocyst numbers at the various concentrations). Potent transmission-blocking activity was observed for the new compounds, with IC50 values for reduction of the intensity of infection being 507.3, 61.6 and 413.3 nM, respectively, (Fig. 3 and Supplementary Fig. 6). We have

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**Table 1 Compound potency in kinase and cell-based assays**

| Compound name | IC50 WT (nM) (n =) | IC50 T618Q (nM) (n =) | EC50 WT (nM) (n =) | EC50 T618Q (nM) (n =) | EC90 WT (nM) (n =) | EC90 T618Q (nM) (n =) |
|---------------|-------------------|----------------------|-------------------|---------------------|------------------|----------------------|
| ML1           | 3.1 +/-0.22 (68)   | 8440 +/-1070 (14)    | 395.0 +/-21.9 (82)| 5952 +/-594.7 (14) | 696.8 +/-98.9 (82)| 20,715 +/-3209 (14) |
| ML2           | 1.71 +/-0.22 (2)   | 1713 +/-218.5 (2)    | 196.6 +/-13.4 (6) | 140.2 +/-12.0 (3)  | 627.6 +/-125.8 (6) | 955.7 +/-58.2 (3)    |
| ML3           | 0.79 +/-0.15 (2)   | 842 +/-74.9 (2)      | 63.4 +/-3.7 (9)   | 195.1 +/-8.1 (3)   | 235.5 +/-18.7 (9) | 278.7 +/-29.4 (3)    |
| ML4           | 0.45 +/-0.04 (2)   | 231 +/-9.1 (2)       | 25.3 +/-1.7 (12)  | 106.7 +/-12.2 (3)  | 106.6 +/-13.4 (12)| 185.8 +/-26.2 (3)    |
| ML5           | 0.88 +/-0.04 (2)   | >100,000 (4)         | 329.1 +/-3.5 (6)  | 12,735 +/-1519 (3) | 445.2 +/-2.5 (6)  | 61,465 +/-1542 (3)   |
| ML6           | 0.13 +/-0.02 (2)   | 52,500 +/-1000 (2)   | 102.3 +/-13.4 (6)| 1855 +/-147.5 (3)  | 141.5 +/-12.2 (6) | 3491 +/-822 (3)      |
| ML7           | 4.34 +/-0.04 (2)   | >100,000 (2)         | 488.9 +/-39.4 (6)| 35,111 +/-2739 (3)| 633.3 +/-15.8 (6)| 60,663 +/-2931 (3)   |
| ML8           | 0.79 +/-0.01 (2)   | 73,730 (4)           | 148.1 +/-10.6 (9)| 5574 +/-474.1 (3)  | 181.6 +/-8.6 (9) | 31,022 +/-2243 (3)   |
| ML9           | 1.19 +/-0.07 (2)   | >100,000 (2)         | 104.6 +/-5.3 (6)  | 6900 +/-9221 (3)   | 158.1 +/-6.8 (6) | 48,327 +/-1790 (3)   |
| ML10          | 0.16 +/-0.01 (2)   | 29,540 +/-519 (2)    | 2.1 +/-0.2 (18)   | 2430 +/-413.3 (6)  | 4.50 +/-0.5 (18)  | 4612 +/-1549 (6)     |

Compounds were tested for their ability to inhibit the protein kinase activity of the full length 6His-tagged PfPKG recombinant protein (IC50). This was carried out using a microfluidic mobility shift assay, which measures the separation of phosphorylated/non-phosphorylated forms of a fluorescent peptide substrate to evaluate conversion. Hypoxanthine incorporation assays were then used to measure the ability of the newly synthesized compounds to inhibit P. falciparum (3D7 and T618Q clonal lines) asexual blood stage growth in vitro (EC50, and EC90). Data are in nM ( +/- the s.e.m.). The number of biological replicates (carried out in triplicate) is shown in brackets.
previously shown that PKG is essential for gametocyte activation and transformation into gametes\(^\text{15}\), and propose that inhibition of these events underlies the observed transmission-blocking activity. To confirm the mechanism of action of ML10 in gametocytes, we compared the ability of ML1 and ML10 to block rounding up of \(P. \text{falciparum}\) wild-type (3D7) and gatekeeper mutant (T618Q) stage V gametocytes. Both compounds blocked rounding up of wild-type gametocytes, but the majority of T618Q gametocytes were able to round up in the presence of both PKG inhibitors showing that they are insensitive to the compounds and thereby confirming that the primary target of ML10 (and ML1 as previously shown\(^\text{15}\)) is PKG in gametocytes as well as in blood stages (Supplementary Fig. 7).

**ML10 has little activity against human kinases and cell lines.** To assess the selectivity of ML10, it was tested against a panel of 80 human protein kinases (representing all the main families and including 14 small gatekeeper kinases) and it showed a clean profile with only low levels of inhibitory activity when tested at 100 nM (>600× IC\(_{50}\) against recombinant PIPKG) (Supplementary Fig. 8). Although achieved through an independent experimental approach, this result is consistent with the observed >1100-fold difference in sensitivity between wild-type and gatekeeper mutant parasites reflecting the exquisite specificity of this compound for PIPKG. Of the panel of 80 human kinases, the highest level of inhibition was obtained with human MLK3 (40% at 100 nM). MLK3 has a very low overall similarity to PIPKG with their kinase domains having only 22% identity. Human MLK3 has a large hydrophobic residue (methionine) in the gatekeeper position, which would prevent access of the inhibitor to the gatekeeper pocket and the IC\(_{50}\) for ML10 with MLK3 is >600-fold higher than with PIPKG. The three MLK isoforms most similar to MLK3 are MLK1, MLK2 and MLK4 and these also have bulky hydrophobic gatekeeper residues. Compounds ML1-10 were tested in cytotoxicity assays using HepG2 cells (derived from liver hepatocellular carcinoma). ML10 and most of those tested had EC\(_{50}\) values higher than the maximum dose tested (Supplementary Table 2). To provide additional safety data for ML10, it was tested against three additional human cell lines at concentrations between 0.001–10 μM (\(n=6\); CXR Biosciences Ltd; Supplementary Fig. 9) derived from lung carcinoma (A549), colorectal adenocarcinoma (HT-29), and breast adenocarcinoma (MCF7). For all three lines the EC\(_{50}\) values for ML10 were higher than the highest concentration used (10 μM) confirming a very high selectivity window (>4500 fold) for this compound.

To investigate whether resistance is readily generated to this class of inhibitor using prolonged exposure of parasites to sublethal doses, we followed a published protocol\(^\text{26}\). No resistant parasites were selected, suggesting that substitutions in the compound-binding domain (or at a locus responsible for an alternative resistance mechanism) are not readily selected under these conditions. Drug resistant parasites were, however, readily selected in control cultures treated with atovaquone, but test cultures treated with the PKG inhibitor remained negative for the duration of the experiment (Methods; Supplementary Fig. 10).

**Fig. 1** In vivo efficacy of PKG inhibitors against rodent malaria parasites. a Groups of five female BALB/c mice were infected with 1×10\(^7\)/ml \(P. \text{berghei}\) (ANKA) blood stage parasites in a Peters 4-day test and were given a twice daily dose (25 mg/kg) of one of three test compounds by oral gavage. Chloroquine was used as a positive control at a single daily oral dose of 10 mg/kg. b Groups of five female BALB/c mice were infected with 1×10\(^7\) \(P. \text{chabaudi}\) (AS) blood stage parasites and were given a single oral dose (50 mg/kg) of either ML1 or ML4 by oral gavage just prior to the predicted onset of schizogony. Chloroquine was used as a positive control at a single daily oral dose of 10 mg/kg. c Groups of five BALB/c mice were infected with 1×10\(^7\) \(P. \text{chabaudi}\) (AS) blood stage parasites and were given either a single or twice daily oral dose (50 mg/kg) of ML10 by oral gavage. The first dose was given to both groups of mice just prior to the predicted onset of schizogony and in one group this was followed 3 h later when schizogony was predicted to have been completed. The data are from single experiments each performed on a group of five mice. Error bars show the s.e.m.
Crystal structures give insight into the selectivity observed. To seek insights into the interactions between *Plasmodium* PKG and the new inhibitors, we expressed, purified and crystallized recombinant protein samples from PfPKG and *P. vivax* PKG (PvPKG), but only obtained diffracting crystals from the latter with ML1 (PDB: 5FET) and ML10 (PDB: 5EZR). The two orthologs are 92% identical in sequence over the full length sequences. The kinase domains of the apo structures of the two orthologs, when their atomic positions are aligned (Supplementary Fig. 11), deviate from each other by a negligible root-mean-square distance (RMSD) of 0.3 Å. Clearly, PvPKG is a suitable structural surrogate of PfPKG. We obtained high resolution co-crystals of PvPKG with ML1 and ML10, respectively. The data collected for the resulting co-structures (PDB: 5FET with ML1; 5EZR with ML10) showed clear electron density for each compound. Alignment of their kinase domains with those from the PvPKG apo structure (PDB: 5DYL) and a co-structure with adenylyl imidodiphosphate (AMPPNP), a non-hydrolysable analog of ATP (PDB: 5DZC) resulted in RMSD values lower than 0.3 Å, showing that ligand binding changed the conformation of the protein negligibly (Supplementary Fig. 12). We also note that all ligands formed complexes with the cGMP-free and inactive state of PvPKG. To date, we have not succeeded in our attempts to crystallize full length PKG from either *Plasmodium* species with cGMP bound, with or without inhibitors or other ligands in the catalytic domain. Comparison of the inhibitor-bound structures reveals two key contributors to potency shared by both compounds. First, hydrogen bonds between the amino group in ML10 engaging in hydrogen bonds with D675 (D682 in PfPKG) of the DFG triad (Fig. 4a). Second, the fluorophenyl group occupies the hydrophobic pocket next to the relatively small gatekeeper (T611; T618 in PfPKG) and F676 (F683 in PfPKG) of the DFG triad (Fig. 4a). The latter interaction also confers selectivity, as this pocket would be blocked by the sidechain of a larger gatekeeper residue, such as that found in the T618Q mutant and the majority of S/T kinases (in both humans and parasites). The pocket extends beyond the gatekeeper (Fig. 4b), with the additional cavity unexploited by ML1 but filled by the sulfonamide group in ML10 engaging in hydrogen bonds with D675 (D682 in PfPKG) and F676 (F683 in PfPKG) of the DFG triad (Fig. 4a). Finally, both compounds engage in hydrophobic interactions with a network of residues from both lobes and the hinge of the kinase domain (Fig. 4c and Supplementary Fig. 13). Notably, the
methylation of the imidazopyridine core and the cyclopropylmethylene extension from the aminopyrimidine in ML10 enhances this network and consequently increased the potency of inhibition. Crystallography data collection and refinement statistics are shown in Table 2.

**Discussion**

Currently there is optimism regarding several new chemical entities undergoing clinical development as antimalarials. However, attrition rates in drug development across all therapeutic areas are high. Although the use of protein kinase inhibitors has been a successful strategy for treating a range of human cancers for many years, with 25 approved drugs to date, to our knowledge it has not proved possible to exploit this target class to treat infectious disease. An inhibitor of a *P. falciparum* lipid kinase (PI4K) is showing real promise and is in clinical development. Although our study has focused on the effects of the new PKG inhibitors on blood stages and sexual stages, it has been reported previously that PKG has roles in liver stages and so it would be worth exploring the effects of the new compounds on these additional stages to prevent the initiation of a blood stage infection. Our results suggest that ML10 or an analog with further optimized pharmacokinetic properties, targeting the protein kinase PfPKG, might have significant efficacy in terms of curing malaria in patients as well as blocking transmission within the population in the context of malaria elimination programmes. Selectivity to avoid toxicity and side effects is a major challenge in antimalarial chemotherapy, especially when targeting protein kinases. However, our results with the malaria parasite PKG with its small gatekeeper residue conferring a rare structural feature, have demonstrated that a highly selective inhibitor can be generated with sufficient potency to clear *P. falciparum* infection in vivo. Altogether our results suggest that PKG inhibitors should be considered for development.
development as a component of a future antimalarial combina-
tion treatment.

**Methods**

**Medicinal chemistry strategy summary.** The medicinal chemistry program started with the Merck imidazopyridine, Compound 2, here referred to as ML1 (signifying their generation through a partnership between MRC Technology (now LifeArc) and the London School of Hygiene and Tropical Medicine). Removing the dimethylbenzylamine to 3-methylsulphonylphenyl (ML5) gave similar potency and better microsomal stability to (ML1) but poorer permeability. A further boost to potency came with the addition of a methyl (ML4) to the dimethylbenzylamine. Going back to ML1, changing the 4-fluorophenyl to 3-methylsulphonylphenyl (ML5) gave similar potency and better microsomal stability to (ML1). Through replacement of the 4-fluorophenyl with a phenyl spacer (ML2) improved both cell potency and microsomal stability, but drastically reduced permeability. Reintroduction of the dimethyamine (ML3) further improved potency and restored permeability. A further boost to potency came with the addition of a methyl (ML4) to the dimethylbenzylamine. Going back to ML1, changing the 4-fluorophenyl to 3-methylsulphonylphenyl (ML5) gave similar potency and better microsomal stability to (ML1). Through replacement of the 4-fluorophenyl with a phenyl spacer (ML2) improved both potency and permeability with a slight drop off in microsomal stability. Moving to the 3-methylsulphonamidophenyl (ML7), both potency and perme-
ability were improved (4-fluorophenyl), though replacement of the 4-fluorophenyl with a phenyl spacer (ML2) boosted potency but not permeability. Addition of the carboxy methyl (ML9) again gave a slight improvement in potency and permeability and the addition of the cyclopropylmethene group (ML10) gave a compound with highly potent anti-
malarial activity. Analytical data are shown in Supplementary Note 1.

**Expression and purification of recombinant PKG.** Full length PPGK (NCBI access code accession code XP_001348520) with native codon usage was cloned into the pTrcHis plasmid (Life Technologies) that includes an N-terminal His-tag as described previously. PPGK with threonine 618 replaced with a glutamine (PPGK T618Q) was cloned into the same plasmid as described previously. Recombinant constructs were overexpressed and puriﬁed using a protocol based on that described previously. Briefly, freshly transformed E. coli Rosetta™ 2(DEL) pLyS3 (Novagen; Cat. No. 71403) were used for expression of recombinant PPGK. 500 ml cultures in LB Rich Broth (containing 50 μg/ml carbenicillin and 34 μg/ml chlor- amphenicol) were grown in a shaking incubator at 37 °C until reaching an optical density (OD600) of 0.6–0.7. The temperature was reduced to 16 °C before induction of expression with 1 mM IPTG. Incubation at 16 °C was continued overnight.

The cultures were harvested by centrifugation at 4000g at 4 °C for 30 min, the supernatant removed and the pellet stored at −80 °C for in excess of 1 h. The PKGs were puriﬁed via the histidine tag on the T7 Trap TALON (coblum) columns (GE Healthcare) connected to an AKTA-PF as per the manufacturer’s instructions. Fractions were analyzed by SDS-PAGE and the main peak concentrated on 10 kDa MWCO concentrators (Amicon). Purified proteins were stored in 50% glycerol at −80 °C in single use aliquots. The ﬁnal buffer composition of the puriﬁed product was: 50 mM Tris/HCl pH 7.5, 0.1 mM EGTA, 150 mM NaCl, 0.1% β-mercaptoethanol, 50% glycerol, 0.03% Brj-35, 1 mM benzamidine and 0.2 mM PMSF. PPGK protein preparation was outsourced at the Division of Signal Transduction Therapy, School of Life Sciences, University of Dundee.

**Microﬂuidic assay for recombinant PPGK.** IC50 values were determined for test compounds using a microfluidic mobility shift assay. Briefly, compounds were prepared over a 10-fold dilution series in dimethyl sulfoxide (DMSO) in duplicate in 50 μl volumes using 384-well polypyrrole U-bottomed plates (Thermo Scientiﬁc, UK). The plates contained positive/no inhibitor (DMSO only) and negative (no enzyme) controls in columns 1, 2, and 23, 24. The reaction mix for each well consisted of 20 μl of enzyme/peptide mix (1.25 μM PPGK, 1.5 μM PFMSG, 80 °C for in excess of 1 h. The PKGs were purified via the histidine tag on the T7 Trap TALON (coblum) columns (GE Healthcare) connected to an AKTA-PF as per the manufacturer’s instructions. Fractions were analyzed by SDS-PAGE and the main peak concentrated on 10 kDa MWCO concentrators (Amicon). Purified proteins were stored in 50% glycerol at −80 °C in single use aliquots. The ﬁnal buffer composition of the puriﬁed product was: 50 mM Tris/HCl pH 7.5, 0.1 mM EGTA, 150 mM NaCl, 0.1% β-mercaptoethanol, 50% glycerol, 0.03% Brj-35, 1 mM benzamidine and 0.2 mM PMSF. PPGK protein preparation was outsourced at the Division of Signal Transduction Therapy, School of Life Sciences, University of Dundee.

**Pharmacological assay for recombinant PPGK.** To allow dosing to be carried out more conveniently during the day, reversal of the light/dark exposure of mice for 2 weeks prior to the experiment was used to switch schizogony from midnight to noon each day. Mice were dosed at different time points in an attempt to have maximal coverage at the point of schizogony. All animal work protocols carried out at LSHTM were approved and licensed by the United Kingdom Home Ofﬁce as governed by law under the Animals (Scientific Procedures) Act 1986, in strict accordance with the Code of Practice Part 1 for the housing and care of animals (21 March 2005), available at http://www.homeofﬁce.gov.uk/science-research/animal-research/

**Efficacy against *Plasmodium falciparum* in vivo.** The efﬁcacy of ML10 against the *P. falciparum* 3D7 line was tested in the GSK *P. falciparum* humanized mouse model (GSKPfHuluMouse)22. The uncloned PPGK 3D7 line was kindly donated by Drs. E. De-Cas and L. Delhaes from Institut Pasteur (Lille, France) and was adapted to grow in peripheral blood of engrafted NOD scid/γc-null mice (The Jackson Laboratory, Bar Harbor, ME) were engrafted with humanized erythrocytes (Red Cross Transfusion Blood Bank in Madrid, Spain) by intraperitoneal daily injection with 1 ml of a 50% hematocrit erythrocyte suspension (RPMI 1640 medium, 25% (vol/vol) decellularized human serum, 3.1 mM hypoxanthine) throughout the experiment. The mice were infected with 2 × 107 *P. falciparum* PfD709 (3D7 CC) infected erythrocytes (Day 0) at ~ 40% chimerism in peripheral blood. The drug treatment was given to mice on Day 3 after infection every 12 h for 4 consecutive days by oral gavage in a volume of administration of 10 ml/kg bodyweight, at a dose of 50 or 100 mg/kg bodyweight. ML10 was prepared in 90% dH2O, 7% Tween 80, 3% ethanol. Parasitemia was measured by flow cytometry in samples of peripheral blood stained with the fluorescent nucleic acid dye SYTO-16 (Molecular Probes, Cat. No.: S-7578) at a concentration of 5 μM and anti-murine erythrocyte TER119 monoclonal antibody (10 μg/ml; Beckton Dickinson) in serial 2 μl blood samples taken every 24 h until assay completion as described22. AUCEmax is the average daily exposure of the compound in whole blood that reduces parasitemia at day 7 of the in vivo assay by 90% with respect to parasitemia in vehicle-treated mice.

The plasma levels of ML10 in mice from the efﬁcacy experiment were measured in serial samples of peripheral blood (25 μl) taken by tail puncture at 0.25, 0.5, 2, 4, 6, and 12 h after the first administration. The blood samples were immediately lysed with 25 μl of 0.1 M sodium citrate, frozen at −80 °C until analysis. The compounds were extracted from 10 μl of each lysate by liquid–liquid extraction in the MultiScreen Solvinert 0.45 μm Hydrophobic PTFE 96- well plate system (Millipore) and stored frozen at −80 °C until analysis by LC/ MS/MS in API 4000 (AB Sciex, Framingham, MA). The compound concentration vs. time data were analyzed by non-compartmental analysis using Phoenix® Version 6.3 (Pharsight Corporation, Mountain View, CA, USA). Additional statistical analysis was performed with GraphPad Prism® Version 6.02 (GraphPad Software Inc, San Diego CA, USA).

All the experiments were approved by the DWD Ethical Committee on Animal Research. Animal work was performed at the DWD Laboratory Animal Science facilities accredited by AAALAC, and conducted in accordance with European Directive 86/609/EEC and the GSK Policy on the Care, Welfare and Treatment of Animals. The human
biological samples were sourced ethically and their research use was in accord with the terms of the informed consent. Erythocyte concentrates from malaria-negative donors were provided by Biobancos de Castilla y Leon, Barcelona and Centro de Transfusiones de Madrid and the Red Cross Transfusion Blood Bank in Madrid, Spain. Research was conducted according to POL-GSK-410 and was in accord with the terms of the informed consent of each donor.

**In vitro parasite reduction ratio.** In vitro PRR testing was conducted at GlaxoSmithKline (Tres Cantos, Madrid, Spain) as previously described. The assay used the limiting dilution technique to quantify the number of parasites that remained viable after drug treatment. *P. falciparum* strain 3D7A (Malaria Research and Reference Reagent Resource Center (MR4), BEI Resources, Cat. No. MRA-102) was used for this experiment. Drug concentration corresponding to 10× IC50 of parasites exposed to treatment were identical to those used at GSK in the IC50 determination (2% hematocrit, 0.5% parasitemia). Parasites were treated for 120 h. Drug in culture medium was renewed daily over the entire treatment period. Parasite samples were collected from the treated culture every 24 h (24, 48, 72, 96, and 120 h time points); drug was washed out of the sample, and parasites were cultured drug-free in 96-well plates by adding fresh erythrocytes and culture medium. To quantify the number of viable parasites after treatment, threefold serial dilutions were used with the above-mentioned samples after removing the drug. Four independent serial dilutions were performed with each sample to correct experimental variations. The number of viable parasites was determined after 21 and 28 days by counting the number of wells with growth using [3H]-hypoxanthine incorporation. The number of viable parasites was back-calculated by using the formula

\[
\text{IC}_{50} = \frac{\ln \left(1 - \frac{\text{no of viable parasites}}{\text{no of wells}}\right)}{t 
\]

where \( n \) is the number of wells able to render growth and \( X \) the dilution factor (when \( n = 0 \), number of viable parasites is estimated as zero).

**In vitro selection of resistant parasites.** The resistance profiling was carried out according to the method described previously, which looks for the emergence of resistant mutants against a compound concentration of 3× IC50. *P. falciparum* lab isolate D2 (from stocks held at LSHTM) was used for this experiment and the control compound used was atovaquone. Parasites were plated in triplicate at 107 parasites/well and maintained until parasites were observed microscopically (using Giemsa-stained blood films) or until Day 60 whichever was the soonest. Parasites from the atovaquone plate were discarded at Day 18 and cultures treated with the PKG inhibitor ML1 were parasite negative at Day 60. Gametocytes were present initially but were absent from Day 11.

**Standard membrane feeding assays.** Mature *P. falciparum* (NF54) gametocytes (14 day culture, 0.3–0.5% gametocytes, 2% haematocrit) were obtained from an automated tipper system and incubated with compound for 24 h as previously described. Anopheles stephensi (Sind-Kasur Nijmegen strain) were reared at 30 °C and 70–80% humidity. Mature gametocytes were collected on glass slides and smeared on glass slides and air-dried. Gametocytes at stage V and rounded up per 900 erythrocytes were counted three times.

**Gametogenesis assays.** *Plasmodium falciparum* clone 3D7A (obtained from Dr Lisa Ranford-Cartwright, University of Edinburgh) gametocytes were initiated and cultured using an adapted version of the traditional Trager and Jensen method modified by Fivelman and colleagues. Stage V gametocytes were purified on a 60% Percoll gradient. Purified parasite samples were diluted in warm complete media (RPMI-1640 (Sigma), 0.5% Albumax (Invitrogen), 0.03% L-glutamine (Sigma), 25 mM HEPES (pH 7.4)), 10% FCS, 1× GlutaMAX™ Supplement (Life Technologies, 35050061) and 10% human serum. After 2 h, gametocyte cultures were adjusted to 50% hematocrit with human red blood cells and human serum and fed to 3 Anopheles stephensi (Sind-Kasur Nijmegen strain 39 were reared at 30 °C and 70–80% humidity. Blood cells were drawn from a healthy blood donor and enriched for white blood cells using 60% Percoll gradient. Puriﬁcation of gametocytes at stage V and rounded up per 900 erythrocytes were counted three times.

**Cytotoxicity assays with human cell lines.** A549 (lung carcinoma; ATCC, Ref. ATCC® CCL-185™), plated at 7.5x104 cells/well; HT-29 (colonrectal adenocarcinoma; ATCC, Ref. ATCC® HTB-38™), plated at 7.5x104 cells/well; and MCF7 (breast adenocarcinoma; ATCC, Ref. ATCC® HTB-22™), plated at 9x104 cells/well, were placed into clear bottom, opaque 96 well plates in cell line-specific medium and allowed to attach overnight. Cells were incubated with nine concentrations of ML10 (0.0001–10 μM) in MEM Eagle medium (MEM-EBSS with Non-Essential Amino Acids w/o l-Glutamine; Lonza BEI-6262F), 10% v/v FCS, 1× GlutaMAX™ Supplement (Life Technologies, 35050061) and 50 units/ml penicillin and 50 μg/ml streptomycin for 48 h. Six independent assays were performed for each line and were carried out by CXR Biosciences Ltd. End point for cytotoxicity assessment was carried out using an ATP depletion kit (CellTiter-Glo Luminescent Cell Viability Assay for ATP quantitation (Promega, Cat # G7572)). Results expressed relative to the 0.1% DMSO control. Unpaired Student’s t-tests were performed using GraphPad Prism software (7.0).

**Crystallography.** For crystallography, recombinant PvPKG was expressed and purified using a previously described baculovirus system. Crystals were obtained by setting up protein samples in sitting drop vapor diffusion experiments at 18 °C. For ML1, the following crystallization conditions were used: 10% PEG 5000 MME, 5% tascimate, 0.1 M HEPES pH 7.0, 15 mM spermidine, and 25% glycerol. The crystallization conditions for ML10 were: 15.5% PEG 3350, 0.1 M HEPES pH 7, 0.1 M sucinate pH 7.0. For both, data were collected at beam line 19ID of Argonne National Laboratory’s Advanced Photon Source (http://www.sbc.anl.gov/index.html) and processed using HKL-3000 (Table 2). The PvPKG-ML1 co-structure was solved using Phaser for molecular replacement and the previously deposited PvPKG-apo structure (PDB code 5DYL) as a search model. Refinement was carried out using the BUSTER refinement software (version 2.10.0, developed by Briceño et al., 2011 Cambridge, UK: Global Phasing Ltd and REFMAC45) combined with iterative manual model building using the molecular graphics program Coot46 to a final R factor of 21.9 %. The PvPKG-ML10 co-structure was determined by refining the PvPKG apo structure (PDB code 5DYL) against the data collected from the isomorphous complex crystals. The structure was refined using REFMAC45 to a final R factors of 22.5%. The geometry of the final models was checked using MolProbity47 for reasonable clash scores and no ramachandran outliers. Crystallographic details and refinement statistics are summarized in Table 2. The coordinates have been deposited in the Protein Data Bank with the PDB codes 5FET (ML1) and SEZB (ML10).

**Data availability.** Data corresponding to crystal structures presented can be found at the RCSB Protein Data Bank (http://www.rcsb.org/pdb/home/home.do) with the following codes: PvPKG apo: 5DYL, PvPKG with ML1: 5FET, PvPKG with ML10: SEZB. All relevant data are available from the authors upon request.

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**References**

1. WHO, *World Malaria Report 2016* (World Health Organization: Geneva, 2016).
2. WHO, *World Malaria Report 2014* (World Health Organisation: Geneva, 2014).
3. Amarasinghe, C. et al., Dihydroartemisinin-piperazine resistance in *Plasmodium falciparum* malaria in Cambodia: a multisite prospective cohort study. *Lancet Infect. Dis.*, 16, 357–365 (2016).
4. Dondorp, A. M. et al. The threat of artemisinin-resistant malaria. *N. Engl. J. Med.*, 365, 1073–1075 (2011).
5. Neld, H. et al. Evidence of artemisinin-resistant malaria in western Cambodia. *N. Engl. J. Med.*, 359, 2619–2620 (2018).
6. Ashley, E. A. et al. Spread of artemisinin resistance in *Plasmodium falciparum* malaria. *N. Engl. J. Med.*, 371, 411–423 (2014).
7. Sibley, C. H. Observing in real time the evolution of artemisinin resistance in *Plasmodium falciparum*. *BMC Med.*, 13, 67 (2015).
8. Deng, W. et al. The role of two novel regulatory sites in the activation of the cGMP-dependent protein kinase from *Plasmodium falciparum*. *Biochem. J.*, 374, 559–565 (2003).
9. Donald, R. G. et al. Anticoccidial kinase inhibitors: identification of protein kinase Cδ as a cGMP-dependent protein kinase. *Mol. Biochem. Parasitol.*, 149, 86–98 (2006).
10. Garnett, A. M. et al. Purification and molecular characterization of cGMP-dependent protein kinase from *Apicomplexa* parasites. *A novel chemotherapeutic target*. *J. Biol. Chem.*, 277, 15913–15922 (2002).
11. Huang, D. et al. Kinase selectivity potential for inhibitors targeting the ATP binding site: a network analysis. *Bioinformatics.*, 26, 198–204 (2010).
12. Alam, M. M. et al. Phosphoproteomics reveals malaria parasite Protein Kinase G as a signalling hub regulating egress and invasion. *Nat. Commun.*, 6, 7285 (2015).

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13. Brochet, M. et al. Phosphoinositide metabolism links cGMP-dependent protein kinase G to essential Ca2+ signals at key decision points in the life cycle of malaria parasites. PloS Biol. 12, e1001006 (2014).

14. Dvorin, J. D. et al. A plant-like kinase in Plasmodium falciparum regulates parasite egress from erythrocytes. Science 328, 910–912 (2010).

15. Peters, W. The chemotherapy of rodent malaria. XXII. The value of drug-resistant strains of P. berghei in screening for blood schizontocidal activity. Ann. Trop. Med. Parasitol. 69, 155–171 (1975).

16. Vaidya, A. B. et al. Pyrazoleamide compounds are potent antimalarials that target Nα-homostasis in intraerythrocytic Plasmodium falciparum. Nat. Commun. 5, 5521 (2014).

17. White, N. J. et al. Spiroindolone KA6609 for falciparum and vivax malaria. N. Engl. J. Med. 371, 403–410 (2014).

18. Feldmann, A. M. & Ponnudurai, T. Selection of Anopheles stephensi for resistant strains of P. berghei in screening for blood schizontocidal activity. Parasitol. Res. 108, 1559–1566 (2013).

19. Vaidya, A. B. et al. Pyrazoleamide compounds are potent antimalarials that target Nα-homostasis in intraerythrocytic Plasmodium falciparum. Nat. Commun. 5, 5521 (2014).

20. Vaidya, A. B. et al. Pyrazoleamide compounds are potent antimalarials that target Nα-homostasis in intraerythrocytic Plasmodium falciparum. Nat. Commun. 5, 5521 (2014).

21. Baragana, B. et al. A novel multiple-stage antimalarial agent that inhibits parasite egress from erythrocytes. Acta Crystallogr. D. Biol. Crystallogr. 63, 410–414 (2017).

22.植物cGMP-dependent protein kinase (PfPKG): antiparasitic activity of a PKG inhibitor. Proc. Natl Acad. Sci. USA 111, E5455–E5462 (2014).

23. McRobert, L. et al. Gametogenesis in malaria parasites is mediated by the calcium dependent protein kinase 4. Mol. Microbiol. 102, 349–363 (2016).

24. Moon, R. W. et al. A cyclic GMP signalling module that regulates gliding motility in a malaria parasite. PloS Pathog. 5, e1000599 (2009).

25. Collins, C. R. et al. Malaria parasite cyclic GMP-dependent protein kinase plays a central role in blood-stage schizogony. Eurycyt. Cell 9, 37–45 (2010).

26. Govindasamy, K. et al. Invasion of hepatocytes by Plasmodium sporozoites requires GMP-dependent protein kinase. PloS Biol. 6, e139 (2008).

27. Baragana, B. et al. A novel multiple-stage antimalarial agent that inhibits parasite egress from erythrocytes. Acta Crystallogr. D. Biol. Crystallogr. 63, 410–414 (2017).

28. Falae, A. et al. Role of Plasmodium berghei cGMP-dependent protein kinase in parasite egress. Parasitology 146, 691–718 (2014).

29. Moon, R. W. et al. A cyclic GMP signalling module that regulates gliding motility in a malaria parasite. PloS Pathog. 5, e1000599 (2009).

30. Vaidya, A. B. et al. Pyrazoleamide compounds are potent antimalarials that target Nα-homostasis in intraerythrocytic Plasmodium falciparum. Nat. Commun. 5, 5521 (2014).

31. Vaidya, A. B. et al. Pyrazoleamide compounds are potent antimalarials that target Nα-homostasis in intraerythrocytic Plasmodium falciparum. Nat. Commun. 5, 5521 (2014).

32. Vaidya, A. B. et al. Pyrazoleamide compounds are potent antimalarials that target Nα-homostasis in intraerythrocytic Plasmodium falciparum. Nat. Commun. 5, 5521 (2014).

33. Vaidya, A. B. et al. Pyrazoleamide compounds are potent antimalarials that target Nα-homostasis in intraerythrocytic Plasmodium falciparum. Nat. Commun. 5, 5521 (2014).

34. Vaidya, A. B. et al. Pyrazoleamide compounds are potent antimalarials that target Nα-homostasis in intraerythrocytic Plasmodium falciparum. Nat. Commun. 5, 5521 (2014).

35. Vaidya, A. B. et al. Pyrazoleamide compounds are potent antimalarials that target Nα-homostasis in intraerythrocytic Plasmodium falciparum. Nat. Commun. 5, 5521 (2014).

36. Vaidya, A. B. et al. Pyrazoleamide compounds are potent antimalarials that target Nα-homostasis in intraerythrocytic Plasmodium falciparum. Nat. Commun. 5, 5521 (2014).

37. Vaidya, A. B. et al. Pyrazoleamide compounds are potent antimalarials that target Nα-homostasis in intraerythrocytic Plasmodium falciparum. Nat. Commun. 5, 5521 (2014).

38. Vaidya, A. B. et al. Pyrazoleamide compounds are potent antimalarials that target Nα-homostasis in intraerythrocytic Plasmodium falciparum. Nat. Commun. 5, 5521 (2014).

39. Vaidya, A. B. et al. Pyrazoleamide compounds are potent antimalarials that target Nα-homostasis in intraerythrocytic Plasmodium falciparum. Nat. Commun. 5, 5521 (2014).

40. Vaidya, A. B. et al. Pyrazoleamide compounds are potent antimalarials that target Nα-homostasis in intraerythrocytic Plasmodium falciparum. Nat. Commun. 5, 5521 (2014).

41. Vaidya, A. B. et al. Pyrazoleamide compounds are potent antimalarials that target Nα-homostasis in intraerythrocytic Plasmodium falciparum. Nat. Commun. 5, 5521 (2014).

42. Vaidya, A. B. et al. Pyrazoleamide compounds are potent antimalarials that target Nα-homostasis in intraerythrocytic Plasmodium falciparum. Nat. Commun. 5, 5521 (2014).

43. Bezzerra, G. A. et al. Structure of human diphteritopic peptide 10 (DPPT): a modulator of neuronal Kv4 channels. Sci. Rep. 5, 6769 (2015).

44. Minier, W. et al. HKL-3000: the integration of data reduction and structure solution—from diffraction images to an initial model in minutes. Acta Crystallogr. D. Biol. Crystallogr. 62, 859–866 (2006).

45. Murshudov, G. N., Vagin, A. A. & Dodson, E. J. Refinement of macromolecular structures by the maximum-likelihood method. Acta Crystallogr. D. Biol. Crystallogr. 53, 240–255 (1997).