UDP-galactose and acetyl-CoA transporters as \textit{Plasmodium} multidrug resistance genes

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A molecular understanding of drug resistance mechanisms enables surveillance of the effectiveness of new antimicrobial therapies during development and deployment in the field. We used conventional drug resistance selection as well as a regime of limiting dilution at early stages of drug treatment to probe two antimalarial imidazolopiperazines, KAF156 and GNF179. The latter approach permits the isolation of low-fit mutants that might otherwise be out-competed during selection. Whole-genome sequencing of 24 independently derived resistant \textit{Plasmodium falciparum} clones revealed four parasites with mutations in the known cyclic amine resistance locus ($pfcari$) and a further 20 with mutations in two previously unreported \textit{P. falciparum} drug resistance genes, an acetyl-CoA transporter ($pfugt$) and a UDP-galactose transporter ($pfact$). Mutations were validated both in vitro by CRISPR editing in \textit{P. falciparum} and in vivo by evolution of resistant \textit{Plasmodium berghei} mutants. Both $PF\text{ACT}$ and $PF\text{UGT}$ were localized to the endoplasmal reticulum by fluorescence microscopy. As mutations in $pfact$ and $pfugt$ conveyed resistance against additional unrelated chemical scaffolds, these genes are probably involved in broad mechanisms of antimalarial drug resistance.

Malaria is a global health menace, with 3.2 billion people at risk of infection and approximately 438,000 deaths in 2015 (ref. 1). In recent years, mounting evidence of resistance to the artemisinin component of artemisinin-based combination therapies (ACTs), the current standard of care for \textit{Plasmodium falciparum} malaria, has been observed$^{2,3}$. As the emergence of artemisinin resistance threatens recent success in malaria control, it is critical that alternative drugs are developed.

Imidazolopiperazines are promising drug candidates with the potential to aid in malaria elimination. These compounds, in particular KAF156 and the closely related probe compound GNF179 (also known as KAF179), possess low nanomolar potency against \textit{P. falciparum} liver stages, asexual blood stages and sexual stage gametocytes. KAF156 is currently in Phase II clinical trials (Fig. 1a)$^{4,7}$ and could become the first new drug that can prophylactically protect against malaria, eliminate the asexual blood stages that cause disease manifestations and block transmission of intraruralycytic gametocytes to \textit{Anopheles} mosquito vectors. Previous work$^{2}$ has demonstrated that KAF156 exposure can generate resistant parasites with a minimum inoculum for resistance (MIR)$^{9}$ of $1 \times 10^6$ parasites, similar to other preclinical candidates that inhibit PfATP4 (ref. 9) and PfEF2 (ref. 10).

Despite its potential to become a drug that can eliminate all stages of \textit{Plasmodium} infection in human hosts, the mechanism of action of KAF156 remains poorly understood$^{8,11,12}$. Previous work has shown that resistance to KAF156 can be conferred by mutations in the \textit{P. falciparum} cyclic amine resistance locus ($pfcari$)$^{13}$. Here we describe an early dilution in vitro selection approach that enables the isolation of low-fit resistant parasites. We use this method, as well as more conventional approaches, to discover two putative transporters$^{14}$, $pfugt$ and $pfact$. We also show, using genome editing, that variants of both genes confer resistance to KAF156 and GNF179 as well as to unrelated chemical scaffolds. These two transmembrane proteins constitute a previously unreported antimalarial resistance mechanism, distinct from those involving the multidrug resistance-1 (PMDR1)$^{15}$ and the chloroquine resistance transporter (PICRT)$^{15,16}$. The localization of $PF\text{UGT}$ and $PF\text{ACT}$ to the endoplasmic reticulum (ER) offers insights into the mechanism of action of imidazolopiperazines.

**Results**

**Selection of imidazolopiperazine-resistant mutants.** To study the mechanisms of resistance to imidazolopiperazines, we employed a forward genetics approach that included aliquoting the cultures at single-parasite densities into 96-well plates immediately after selection with GNF179 (Fig. 1b). The rationale was that clone isolation concurrent with compound exposure would allow the propagation of low-fit mutants that might otherwise be outcompeted in a mixed culture. A total of 11 putatively resistant clones were obtained from two plates based on this in vitro selection concurrent with compound exposure.

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selection approach: six (A3, A9, B6, D6, E8 and B3) from plate A and five (H5, H8, A2, B5 and D8) from plate B. These initial 11 cloned lines were tested for drug sensitivity to both GNF179 and KAF156. All exhibited a dramatic (22- to 539-fold) increase in resistance compared to the parental Dd2 clone (Table 1).

Genotypic characterization of imidazolopiperazine-resistant mutants. Whole-genome sequencing was performed on the parental P. falciparum Dd2 clone and the 11 resistant clones selected with GNF179. These 12 genomes yielded 2.38 × 10^8 reads of 100 bp with 86% mapping to the P. falciparum reference genome. An average coverage of 59× was obtained with an average of 100 bp with 86% mapping to the P. falciparum genome. An average coverage of 59× was obtained with an average of 100 bp with 86% mapping to the P. falciparum genome.

Figure 1 | Selection of imidazolopiperazine-resistant mutants. a, Chemical structures of GNF179 and KAF156. b, Schematic diagram showing the selection process for spontaneous GNF179-resistant mutants. Briefly, a large inoculum (10^9 parasites) of a clonal line of P. falciparum Dd2 was exposed to GNF179 at 2 × IC99 (38.4 nM) for two complete intraerythrocytic cycles (96 h). After 25 days in culture, wells containing parasites were identified by Giemsa staining and light microscopy. The resistance mutations were then validated using CRISPR/Cas9-based approaches.

Addition of GNF179 (Day 0) → SYBR Green I drug sensitivity assay → 63% Percoll purification → Detection of GNF179 resistant mutants via CRISPR/Cas9 system → Parasite gDNA → Target RNA → PAM (NGG) → Guide RNA → Validation of resistance mutations via CRISPR/Cas9 system

The resistant clones selected with our early dilution cloning method were more resistant than the previously generated resistant clones to KAF156 and GNF179 bearing mutations in pfcari (refs 7,12). Clones from previously reported imidazolopiperazine resistance studies were generated using a stepwise approach in which selection was stopped as soon as resistance emerged. To determine whether mutations in pfact and pfugt would lead to GI50 values of 22- to 539-fold increase in resistance compared to the parental Dd2 clone (Table 1).

Seven of the 11 resistant lines possessed mutations in two genes not previously associated with imidazolopiperazine resistance, namely an UDP-galactose transporter (pfugt) (PlasmoDB ID: PF3D7_1113300) and an acetyl-CoA transporter (pfact) (PlasmoDB ID: PF3D7_1036800) (Fig. 2a). All seven lines mutated in pfugt possessed the same phenylalanine to valine change at position 37 (F37V). One line (B3) acquired a stop-gain mutation at position 242 of pfact (Table 1).

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incorporation and a premature stop-gain mutation at ‘new codons’ 78 and 198, respectively (Supplementary Fig. 1). Overall, these data indicate that mutations in *pfugt* and *pfact* appeared when cultures were exposed to higher GNF179 concentrations.

**In vivo selection of resistant mutants.** To determine whether we would find similar mutations after *in vivo* growth, mice were infected with *Plasmodium berghei* ANKA parasites and, once parasites were visible, were treated with a single GNF179 oral dose at two concentrations, 100 or 30 mg kg\(^{-1}\). After parasite recrudescence, mice were dosed a second time with the same concentration. Resistant parasites were inoculated into a new mouse and infected erythrocytes were collected. Eleven DNA samples (from two mice infected with the parental strain and nine mice harbouring recrudescent parasites) were analysed by whole-genome sequencing. On average, 22 million reads were collected for each sample. Of these, an average of 31.1% aligned to the *P. berghei* ANKA reference genome (with the remaining mapping to the mouse). This sequencing yielded average coverage rates of 32.2-fold (range 6.0–92.9, excluding one failed sample). Alignment of the sequences and comparison to the parent reference sequence showed that six of the seven *P. berghei* samples with good coverage had acquired mutations in the *P. berghei* homologue of *pfact* (PlasmoDB ID: PBANKA_051980). These changes included stop-gain mutations at positions W167, C501, Q244 and Q386, two non-synonymous changes (G511V and T473I) and an intronic change (Fig. 2a). These data suggest that mutations in the acetyl-CoA transporter may be more relevant *in vivo* than mutations in the cyclic amine resistance locus and that the mechanism of resistance to imidazolopiperazines was conserved across parasite species (Supplementary Fig. 2).

**Resistance phenotypes of spontaneous mutants.** GNF179 exhibited low nanomolar potency (5–9 nM) against the laboratory-adapted *P. falciparum* Dd2 strain, which is multidrug-resistant (to chloroquine, quinine, pyrimethamine and sulfadoxine; Table 1) using a 72 h proliferation assay\(^\text{17}\). Representative mutants in *pfact*, *pfugt* and *pfcarl* showed no cross-resistance with standard antimalarial drugs (Fig. 3a). The lines harbouring mutations within either *pfugt* or *pfact* all demonstrated a >tenfold higher level of resistance to GNF179 than any of the observed *pfcarl* mutants. GNF179 exhibited micromolar potency against the seven *pfugt* F37V-mutant lines, a decrease in drug sensitivity in the range of 350–540-fold (Table 1). Despite acquiring a detrimental stop-gain mutation at position 242 in *pfact*, clone B3 remained viable and was highly resistant to GNF179, with an IC\(_{50}\) increase of 353-fold compared to the parental strain (Table 1).

**Membrane topology, homology modelling and distribution of genetic polymorphisms.** TMHMM (ref. 18) was used to predicted membrane topology for the PiCARL, PiUGT, PiACT and PhACT proteins (Fig. 2a). These models predicted all three proteins to be multispanning membrane proteins, with a subset of
resistance mutations in PfACT and PbACT located primarily in cytosolic loops. The truncation mutations resulting from stop-gain codons in the acetyl-CoA transporter would result in the loss of several transmembrane domains and nearly half of the protein. ACT is probably a member of the Major Facilitator Superfamily (MFS), a large and ubiquitous secondary transporter family. The crystal structure of an MFS transporter from the eukaryotic organism Piriformospora indica was used as the template for modelling PfACT and PbACT (Fig. 2b,c). All five non-synonymous amino-acid substitutions (A94T, R108K, S110R, D165N and G559K) in PfACT were situated on the intracellular side of the membrane (Fig. 2b). Except for G559, the four other mutated residues were located at the site corresponding to the intracellular gate also known as the ‘cytosolic tunnel’. The carbons α of stop-gain mutations are represented as yellow balls and labelled. The putative cytoplasmic gating hinge (see text) is indicated.
R108 belongs to Motif-A (G-X3-(D/E)-(R/K)-X-G-[X]-(R/K)-(R/K)), a sequence motif known to play a role in intracellular gating across the MFS transporters. In contrast to PfACT, the two non-synonymous substitutions in PbACT (G511V and T473I) localized to the C terminus, and their functional impact cannot be predicted (Fig. 2a,c). All mutated residues were located in highly conserved regions within the MFS transporters except for the G511 and G599 substitutions (Supplementary Figs 2 and 3).

**Figure 3 | CRISPR/Cas9 mutation validation and localization of PfUGT and PfACT.**

- **a.** IC$_{50}$ values of GNF179-resistant mutants against a panel of antimalarial compounds.
- **b.** Schematic of the pfugt and pfact editing strategy. Cas9 and the target-specific gRNA were provided on a single hdhfr-marked plasmid. For pfugt editing (left), the gRNA was expressed from the T7 promoter, enabled by co-expression of T7 RNA polymerase. For pfact editing (right), the gRNA was expressed from the P. falciparum U6 promoter. The homologous donor sequence was provided on a plasmid expressing the blasticidin S-deaminase (bsd) selectable marker. The donor encoded the putative resistance mutation as well as silent mutations in the gRNA-binding site to prevent cleavage.
- **c.** Comparison of GNF179 IC$_{50}$ values for the drug-selected and CRISPR-generated parasite lines.
- **d, e.** Live-cell imaging of parasites expressing mRFP-PfUGT and the ER marker GFP-PfSec12 (d) or PfACT-GFP (e) and stained with the ER marker ER-tracker red. Both PfUGT and PfACT were located in a perinuclear pattern characteristic of the parasite ER. Nuclei were stained with Hoechst 33342. Scale bar, 5 µm. In a and c, error bars represent standard deviation (s.d.) from three biological experiments with technical duplicates.
chance, a few additional noncoding variants were found in the sequencing data. To demonstrate that pfugt and pfact mutations were sufficient to confer GNF179 resistance, we introduced the pfugt F37V and pfact S242* mutations into a wild-type (WT) Dd2 strain using the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system. Sanger sequencing of both edited loci revealed the presence of the resistance mutations as well as the silent mutations engineered into the Cas9-cleavage sites (Supplementary Fig. 4). Introduction of the F37V mutation into the pfugt gene resulted in a 183-fold increase in resistance to GNF179 (926.4 versus 5.1 nM for the parental Dd2 line), similar to the expected value (2.3 µM) observed with the spontaneous pfugt stop-gain mutant (Table 1 and Fig. 3c). As with the evolved clones, the edited clones did not show cross-resistance to artemisinin (Table 1 and Fig. 3a). Taken together, our results showed that the SNVs observed in pfact and pfugt were sufficient to confer resistance to GNF179.

The proteins encoded by both pfugt and pfact are predicted by the Malaria Parasite Metabolic Pathways database to reside on the ER/Golgi membrane. To localize PFUGT, we generated a transgenic parasite line expressing a second copy of pfugt fused with mRFP. Live cell imaging revealed a perinuclear distribution of mRFP-PFUGT, characteristic of the ER. This localization was confirmed by co-transfection of an ER marker, GFP-PfSec12 (ref. 24), which showed nearly perfect co-localization with mRFP-PFUGT (Fig. 3d). Live cell imaging also revealed a similar subcellular localization of the evolved mutants with premature stop codons in pfact, although these were able to tolerate high levels of GNF179 (IC50 values between 895 nM and 7.9 µM). Introduction of the S242* truncation mutation into pfact yielded a 947-fold decrease in sensitivity to GNF179 (IC50 = 4.8 µM versus 5.1 nM for the parental Dd2 line), similar to the expected value (2.3 µM) observed with the spontaneous pfact stop-gain mutant (Table 1 and Fig. 3c).

Figure 4 | GNF179-resistant mutants display poor fitness. a,b. Competitive growth kinetics of PIUGT (a) and PIACT (b) mutants, showing the ratio of the PIUGT or PIACT mutants versus the parental Dd2 strain over ten generations, as quantified using the droplet digital polymerase chain reaction (DDPCR). Three different starting ratios of mutant:WT (1:10, 1:1 and 1:10) yielded similar results, with the WT line outgrowing the mutant lines. Error bars (s.e.) represent technical duplicates for a biological experiment. c. Growth at 96 h, expressed as mean parasitaemia of the PIUGT and PIACT mutants, or the WT Dd2 strain, when grown in independent flasks, either in the absence of drug pressure (n = 4) or in the presence of GNF179 at 2 × WT IC50, that is, 38.4 nM (n = 2) for a biological experiment. Parasitaemias were measured using flow cytometry. Percent parasitaemias were significantly different in the presence of GNF179 for the PIUGT mutant (*P = 0.0066 < 0.05, t-test, two-sided) and PIACT mutant (**P = 0.0050 < 0.01, t-test, two-sided), despite both mutants having an IC50 >50-fold higher than the concentration used. d. PIUGT and PIACT mutants cultured in the absence of GNF179 for four months demonstrated no phenotypic reversion in drug susceptibility (P > 0.05) (n = 4). e. Giemsa-stained morphology of PIUGT and PIACT mutants (GNF179 IC50 >2 µM for both lines), cultured in the presence of 38.4 nM GNF179 as in c. Mutant parasites appeared less organized in the distribution of merozoites in the schizont stage, but ring and early trophozoite stages remained similar to that of the WT strain. Scale bar, 5 µm. In c and d, error bars represent standard error (s.e.).
distribution of PfACT-GFP, which co-localized with the ER-specific cellular stain ER-tracker red (Fig. 3e).

**PfUGT and PfACT mutations possess an associated fitness cost.** One feature of the early dilution selection protocol (Fig. 1b) is that the serial dilution is upfront, limiting the probability of having fitter clones outgrow weak ones. We observed, during routine culturing, that parasites encoding a *pfugt* or *pfact* mutation grew at a slower rate than the parental strain or the *pfcarl* mutant lines. To document growth rates more quantitatively, we performed continuous culturing of mixed ratios of mutant and parental parasites over a period of ten generations. This competitive growth experiment revealed that *pfugt-F37V* and *pfact-S242* mutants possessed a higher fitness cost than WT Dd2 (Fig. 4a–b). We performed individual growth kinetic analysis using flow cytometry to monitor the parasitaemia of these lines in the presence and absence of GNF179. In the absence of compound pressure, the growth rates of the *pfugt-F37V* and *pfact-S242* lines were similar to each other (Fig. 4c). The presence of GNF179 imposed a stress on the growth of the mutant lines, with parasitaemia decreasing to one-third to one-half that of the drug-free counterparts (Fig. 4c). Regardless of the presence of the GNF179, both the *pfugt-F37V* and *pfact-S242* mutants had impaired growth compared to the parental Dd2 strain. Despite this fitness cost, representative resistant lines subsequently cultured in the absence of GNF179 for four months retained similar levels of resistance, indicating these mutations still permit stable parasite growth *in vitro* (Fig. 4d). Examination of Giemsa-stained blood smears of the mutant parasite lines during routine culture, maintained in the presence of 38 nM GNF179 to prevent possible reversion, revealed morphological changes in both the *pfugt* and *pfact* mutant isolates, in particular during the late trophozoite to schizont stages (Fig. 4e). This suggests that PfUGT and PfACT could play an important role in late trophozoite and schizont development.

**Cross-resistance against a panel of imidazolopiperazine analogues.** Given that both *pfugt* and *pfact* appear to be transporters based upon their sequence homologies, we hypothesized that GNF179 resistance might be due to transporter activity, rather than representing direct targets of GNF179. To test this hypothesis, we examined whether the mutant forms of these genes also conferred resistance to additional chemical compounds. We first evaluated the *pfugt-F37V* and *pfact-S242* evolved resistant lines for cross-resistance against a panel of 62 imidazolopiperazine chemical analogues. These analogues incorporated various substitutions at the R1 to R5 positions on the imidazolopiperazine core (Fig. 5a). Twenty-six imidazolopiperazine analogues were inactive against the WT strain and proved equally inactive against the *pfugt* and *pfact* mutants. A bulky substitution on both R1 and R2 generally resulted in a lack of activity (IC$_{50}$ >10 µM) against all tested strains. Among the 36 analogues with activity against WT parasites, none could overcome the resistance phenotype of the *pfugt* and *pfact* mutant lines, although some variability was observed in the overall level of resistance. The stop-gain (S242*) mutation in the acetyl-CoA transporter rendered this parasite highly cross-resistant to all the chemical analogues tested, except for compound 13 and KAF156, which retained relatively good sub-micromolar potency (Fig. 5a). We note that the *pfact-S242* mutant was slightly more sensitive to KAF156 than to GNF179, with a lower fold-shift in the 10–100× range.

**Mutant lines show cross-resistance against non-imidazolopiperazine scaffolds.** We next tested whether the *pfugt-F37V* (mutants A9 and B5) and *pfact-S242* (mutant B3) resistant lines demonstrated cross-resistance against other antimalarials, defined as a greater than fivefold increase in the mean IC$_{50}$ value, against a panel of compounds of unrelated scaffolds (Fig. 5b,c). A total of 69 compounds were evaluated, comprising a panel of 10 standard antimalarials and 59 additional Novartis compounds inclusive of KAF156 and GNF179. Thirteen compounds were active only or primarily against the parental strain, with evidence of cross-resistance in the mutant lines. These compounds included GNF156 and KAF179, as discussed in the following (Fig. 5b), and another nine that will form part of a future study. A further two compounds (compounds 5 and 6) showed increased potency against the mutant lines (Fig. 5b).

Four compounds displayed differential cross-resistance among the parasite lines. Compound 3 (Tyroscherin), a known inhibitor of insulin-like growth factor (IGF)-1 dependent growth and structurally unrelated to GNF179 and KAF156, demonstrated cross-resistance in both *pfugt* and *pfact* mutants with 30- to 50-fold IC$_{50}$ increases (Fig. 5c). Compound 4, belonging to the oxazole class, was active against the *pfact* mutant but displayed a loss in potency against the *pfugt* mutant. In contrast, compounds 5 (KDU691) and 6, both lipid kinase inhibitors, demonstrated a modest increase in potency against both mutant lines (Fig. 5b and Supplementary Table 1). Recent publications have also demonstrated pcarl as a potential multidrug resistance mechanism. The cross-resistance data presented here on *pfugt-F37V* and *pfact-S242* mutants as well as on *pfcarl* (ref. 13) suggest that these three proteins (PICARL, PFUGT and PFACT) could participate in a common resistance mechanism.

**Discussion** Although *in vitro* evolution and whole-genome analysis is often used to discover putative targets of small molecules identified in antimalarial cellular screens, it is equally useful for characterizing potential pathways of resistance. In this study, we present *pfugt* and *pfact* as multidrug resistance genes in *Plasmodium*. Although their modification resulted in a significant loss of parasite fitness, their integrity should nevertheless be monitored in field studies involving imidazolopiperazines or other novel antimalarial compounds undergoing development that do not act against traditional antimalarial targets such as PDHFR, β-haematin or cytochrome bc1.

Here, we provide definitive evidence that mutations in *pfact* and *pfugt* confer resistance to imidazolopiperazines, as well as other scaffolds. More work will be needed to establish their cellular role. The fact that both are predicted to encode transporters and that *pfact* is non-essential argues that neither is a target of KAF156. Both predicted proteins are members of the MFS, the largest and most ubiquitous secondary transporter family responsible for the translocation of small molecules including metabolites, nucleosides, oligosaccharides, amino acids, oxyanions and drugs. MFS transporters have been established as drug-specific or multidrug efflux pumps in bacteria and fungi.

All members of the SLC35A2 (UDP-galactose translocator) family bear Pfam domains belonging to eukaryotic triose phosphate transporter (TPT), the nucleotide-sugar transporter (NST) families and the drug/metabolite transporter superfamily (DMT)22. Interestingly, the *P. falciparum* chloroquine resistance transporter (PICRT) is also a member of DMT, but is localized to the digestive vacuole. NSTs have been associated with drug resistance in organisms such as Candida albicans and Saccharomyces cerevisiae. In humans, mutations in SLC35A2 cause a congenital disorder of glycosylation. Although further work is required to understand the native function of PfUGT in *Plasmodium*, the ER localization is consistent with a function in transporting UDP-galactose from the cytosol into the ER where it may play a role in post-translational modification of proteins.

The function of PfACT in *P. falciparum* is predicted only by homology. Evolutionarily, it is orthologous to a human AT-1 protein (also...
Figure 5 | Cross resistance against a panel of imidazolopiperazine analogues and unrelated antimalarial compounds.  

a, Heat maps of biological activity (pIC₅₀) of 62 imidazolopiperazine analogues (median of two biological experiments with technical duplicates) against GNF179-resistant mutants. No association was observed between R-group descriptors (volume and lipophilicity) and the pIC₅₀ activity profiles (defined as −log₁₀(IC₅₀)) in the heat maps.  

b, Structures of non-imidazolopiperazine scaffolds.  

Activity profiles of non-imidazolopiperazine scaffolds in terms of fold-change in IC₅₀ compared to WT. Each colour represents a different compound. Three biological experiments were performed with technical duplicates. Error bars represent s.d.
known as SLCS3A1). Heterologous expression of its cDNA in COS-1 cells allows these cells to transport radio-labelled acetyl-coA and to form O-acetylated (Ac) gangliosides58, a process that occurs in Golgi vesicles59. In addition, AT-1 regulates the acetylation status of ER-transiting proteins in human cells60 and is also associated with neurodegenerative disorders61. Many of the identified mutations are predicted to localize to the cytoplasmic side of PFACT, suggesting that they prevent KAF156 from entering the ER or Golgi.

The observations that mutations in pfact and pfact, as well as pfcarl, which encodes a Golgi-localized protein13, convey resistance to a variety of imidazolopiperazine compounds as well as unrelated scaffolds provides clues about the mechanism of action of KAF156/ GNF179 and other small molecules whose activity can be modulated through mutations in these genes. Our hypothesis is that PFACT/PUGT may be involved in transporting compounds into the ER or Golgi, where they may act on cellular processes such as membrane trafficking or post-translational modification. Interestingly, KDU691, another antimalarial candidate that targets PfPI4K, a phosphatidylinositol-4-kinase, affects membrane trafficking from the Golgi by depleting phosphatidylinositol-4-phosphate lipid pools32. Furthermore, the PfPI4K orthologue in Saccharomyces, Pkl1, is localized to the Golgi where it regulates secretion and yeast temperature-sensitive Pkl1 mutants can be complemented by the Plasmodium enzyme13,34. The cellular targets of other small molecules, including KAF156, are not known but tyroscherin contains a hydrocarbon tail and, in C. albicans, downregulation of ergosterol biosynthesis genes sensitizes cells to its activity65. Although our data set is small, it is interesting to note that compounds that seem to be affected by these pathways are more likely to have activity in other stages of the lifecycle—both KAF156 and KDU691 have potent activity against stage V gametocytes and parasite liver stages, indicating that they do not act through haemoglobin digestion, or DNA replication. These data suggest that pfact and pfact could become more important as drug discovery efforts shift away from compounds that act mostly against asexual blood stage parasites, for example, by inhibiting haemoglobin digestion.

Although the dramatic decrease in drug sensitivity caused by these mutations raises concerns that imidazolopiperazines might succumb to drug resistance in the field, parasites that harbour mutations in these genes proliferate at a reduced rate. Their low fitness might not survive the competition for nutrients in a polyclonal environment such as the human body, or may be more readily cleared by the immune system. Furthermore, while pfact is not essential for blood stages, it could be essential for other stages of the lifecycle, and the mutations might not be transmitted, as has been recently shown for atovaquone resistance66. Nevertheless, it will be critical to select an adequate combination partner for KAF156 as it enters Phase III clinical trials.

Methods

P. falciparum culture. The parental P. falciparum Dd2 strain and all GNF179-resistant mutants were cultured under standard conditions67. The Dd2 strain is resistant to chloroquine, quinine, pyrimethamine and sulfadoxine and replicates at a rate of five to sixfold per 48 h generation in vitro68. Single donor human O+ whole blood (Innovative Research) was obtained from Novi. Leukocyte-free erythrocytes were stored at 50% haematocrit in RPMI 1640 washing medium.

Determination of parasitemia. A thin blood smear was fixed with 100% methanol (Fisher Scientific) for 1 min before staining in 10% vol/vol Giemsa (Sigma-Aldrich) in phosphate-buffered saline for 15 min. Giemsa is used to visually distinguish P. falciparum parasites from surrounding uninfected erythrocytes. Parasitemia was determined from Giemsa-stained slides under a light microscope (Nikon YS100) with x100 magnification.

Selection of imidazolopiperazine-resistant P. falciparum mutants. A clonal P. falciparum Dd2 parasite line was cultured at 37 °C in complete RPMI medium at 4% haematocrit in an incubator with reduced oxygen environment.23 The IC50 of GNF179 was determined to be 9.0 nM using a 72 h SYBR Green I drug sensitivity assay.23 Prior to selection, an aliquot of the parental line was stock stored as a reference for subsequent whole genome sequencing analysis. A single-step selection to pre-existing resistant mutants was employed by the addition of GNF179 at greater than fully inhibitory concentration (2 × IC50, 38.4 nM) to a population of 1 × 108 parasites (Fig. 1b). Excessive debris produced by the death of drug-sensitive parasites was removed using a 63% Percoll purification step,26 performed four days post exposure to GNF179. The schizont-containing, interface and pellet fraction containing ring and trophozoite-stage parasites were pooled together and re-suspended in 100 ml of culture media, adjusted to 2.5% haematocrit, dispensed into 96-well plates and maintained under 2 × IC50, drug pressure for 25 days. Negative control wells that contained culture at 2.5% haematocrit without any parasite were also present in each plate. Parasites were kept under constant drug pressure with replacement of complete media, washed blood and drug every other day. After 25 days in culture, mutants were selected microscopically using Giemsa-stained slides.

Additional selections were performed using either the traditional ‘ramp-up’ selection method or the high-concentration selection method. For the ramp-up selection method, representing selection S2 in Table 1, D37 strain parasites were cultured under normal conditions while simultaneously treated with gradually increasing doses of GNF179 for 2 months (starting at 5 nM and ending at 40 nM), with cultures monitored via Giemsa-strained thin smears every day, until parasite cultures exhibited at least 5% resistance versus WT. Parasites were cloned by limiting dilution and DNA was extracted for whole-genome sequencing. For the high-concentration selections (selection S1 D37 and Dd2 in Table 1), both D37 and Dd2 parasites were exposed to 50 nM GNF179 for 10 days. GNF179 was removed and parasite cultures were allowed to recover under normal culturing conditions. Parasites were then cloned via limiting dilution.

Isolation and determination of splicing variants in pfact. Both WT D37 and S2-D37-1B and S2-D37-2C parasites were cultured as indicated above. Parasites were washed with 1 × cold PBS, treated with 0.15% saponin, washed twice more with 1 × cold PBS, and total RNA was extracted from asynchronous parasites using the Qagen RNeasy mini kit. cDNA was synthesized using random hexamers and superscript II (Life Technologies) at 42 °C for 2 h. Regions of PFACT were amplified with the primers indicated in Supplementary Table 3 via PCR, then examined via Sanger sequencing.

Selection of imidazolopiperazines-resistant P. berghei-ANKA mutants. The P. berghei ANKA strain was a gift from C. Jensen, Leiden University Medical Center, Netherlands. GNF179-resistant P. berghei ANKA mutants were selected in four- to eight-week-old female BALB/c mice (n = 18). Briefly, ten mice were infected through the tail vein with 1 × 106 P. berghei ANKA parasites from a single batch and treated with either 30 mg kg–1 (n = 4) or 100 mg kg–1 (n = 4) GNF179 or were untreated (n = 2). Parasites extracted from all eight treated mice were re-infected into eight naive mice for propagation and isolation of infected reticulocytes for DNA preparation. For the purpose of in vivo mutant selection of P. berghei parasites we did not require a statistically significant number of animals. Animals were neither randomized nor blinded. Parasites were enumerated by thin blood smear and quantified using line intersect methods70. The formula used for GNF179 was 10% ethanol, 30% PEG400 and 60% vitamin E α-tocopherol polyethylene glycol 1000 (TPGS) (10% solution) and was administered by gavage. The first treatment was initiated at a level of parasitaemia of 4–10%, left to drop below the limit of detection, and repeated only when recrudescence parasites once again reached 4–10% parasitaemia. This was repeated until the level of parasitaemia no longer decreased. Surviving parasites were transfused into naive mice under either 30 or 100 mg kg–1 daily treatment with GNF179, and the growth kinetics were monitored every two days by sampling and Giemsa staining. Parasites were collected by cardiac puncture at 4% parasitaemia, passed through a filter to remove white blood cells before DNA isolation, and sequenced as described for P. falciparum. Ethical approval for the study was obtained from the Animal Care and Use Committee at the Novartis Institute for Tropical Diseases.

Drug sensitivity assay using SYBR Green I. Drug susceptibility was measured using the malaria SYBR Green I-based fluorescence assay37. Each culture was tested in duplicate on a ten-point concentration curve prepared by a threefold dilution from 10 μM to 0.5 nM. At least five independent experiments were carried out for IC50 determinations of KAF156- and GNF179-resistant mutants. IC50 values were calculated using a nonlinear regression curve fitted in GraphPad Prism 5.0.

Parasite extraction and genomic DNA isolation. Cultures were scaled up to at least 4–5% parasitaemia in 100 ml RPMI medium at 2.5% haematocrit before parasite extraction from the red blood cells. The cultures were reconstituted to 10% haematocrit with fresh RPMI medium and transferred to a 15 ml falcon tube. Lysis buffer (1.5% w/v sodium citrate and 1.0% w/v bovine serum albumin in PBS) was added on ice in ten pellet volumes. Following lysis of red blood cells, indicated by a clear red supernatant, the lysed cultures were centrifuged at 2,700g for 5 min at 4 °C. The supernatant was removed by aspiration and the cells were washed twice using chilled PBS in microcentrifuge tubes. Cell pellets were stored at −80 °C until genomic DNA was isolated using a Blood and Cell Culture DNA Mini Extraction Kit (Qagen, cat. no. 13323).
Whole-genome sequencing. DNA libraries for each gDNA sample were prepared for sequencing using the Nextera XT kit (Illumina, cat. no. FC-131-1034) following the standard dual index protocols. Libraries were clustered and run on an Illumina HiSeq 2500 using the RapidRun mode, sequencing 100 base pairs in depth on either end. Paired-end reads were either aligned to the *P. falciparum* 3D7 reference genome (PlasmoDB v. 11.0) or the *P. berghei* ANKA reference genome (PlasmoDB v 9.0), as previously described13,14. Because *P. berghei* samples were unable to be cloned using limiting dilution methods, the sample analysis for these samples included mutations with mixed read ratios (*pfug* mutations W167*, Q244*, Q386* and T473*). To identify possible CNVs, the interval depth for each gene was calculated using GATK Diagnostic Targets. To reduce noise levels, intervals were normalised across each sample and analysed against four 3D7 parent sample sequences previously in our laboratory. Finally, samples were compared to their respective parents, and normalized interval depth ratios >2 were flagged. All SNVs were visually confirmed using the Broad Institute web-based sequence browser IGV. The NCBI short-read access library for the *P. falciparum* GNF179 were uploaded under study accession no. SRP072010. The additional compounds13 of various scaffolds using the SYBR Green I drug sensitivity assay as described above. Representative resistant lines were tested for cross-resistance against a panel of 59 drugs using the SYBR Green I drug sensitivity assay as described above. The guide RNA sequence (listed as gRNA + PAM) used for *PF3D7_1113300* was GATTTTCAAGTCATGTGTAAGG, while the guide RNA sequence for *PF3D7_1036800* was GAAAAGATTTTTAAATCTGG. Donor templates consisted of the full-length coding region (1,032 bp) for the UDP-galactose transporter and a 1 kb fragment centred on the mutation for the Acetyl-CoA transporter. Mutations in the donor template, both for the gRNA target site and the desired SNV, were introduced using the Quikchange II kit (Agilent Technologies). Primers for the donor templates are indicated in Supplementary Table 3.

Cross resistance studies. The *P. falciparum* Dd2 parental strain and 11 resistant mutants obtained with the early dilution method were tested for cross-resistance against 62 imidazolopiperazine chemical analogues and 10 standard antimalarial drugs using the 96-well plate format. The full-length coding sequence for the desired SNV was cloned into the CRISPR/Cas9 genome editing plasmid pDC2-PfCAMpro-PfACT-GFP-attP-BSD. This plasmid was transfected into the T98G cell line together with the pINT plasmid to achieve attP × attB integrase-mediated recombination53. The ER was stained with the fungi dye Calcofluor white (Pentax K. K., Tokyo, Japan) and imaged.

Competitive growth kinetics of resistant mutants. *P. falciparum* Dd2 parental strain and the respective mutants (line 79 and replicates for mutant lines in the presence of 38.4 nM GNF179. Six replicates of the parental Dd2 strain were cultured in the absence of drug. A 20 μl sample was taken daily for flow cytometry analysis throughout the course of the experiment, namely at 0, 24, 48, 72 and 96 h. Media were changed at 24, 48, 60, 72 and 84 h. Each sample was stained with 5 μg ml–1 dihydroethidium (Sigma) and 8 nM Hoechst 33342 (Sigma), as previously described52. Flow cytometry analysis was carried out using FlowJo software (Tree Star). Two-sided t-tests at α = 0.05 with equal variances were performed to compare parasitaemia ±GNF179. F-tests were performed to test for the equality of variance (PIUGT: P = 0.375 > 0.05, PIAC-T: 0.230 > 0.05).

DDPCR. DDPCR reactions were performed with 2 ng of genomic DNA template with 2 × droplet PCR supermix for probes (no dUTP). The mix was supplemented with an additional 50 μM dATP/dGTP. Primer and probe concentrations were 900 and 250 nM, respectively. Primers and probes were synthesized by Integrated DNA Technologies. WT probes were 5′-HEX labelled and mutant probes were 5′-FAM labelled. Both probes were 3′-labelled with an Iowa Black Quencher. Emulsions were generated in the DDPCR droplet generator and then transferred to an Eppendorf Twin Tec PCR plate and sealed with a permeable foil seal. Emulsions underwent 40 cycles of amplification in a Bio-Rad C1000 under the following condition: 95 °C for 10 min, a two-step amplification at 94 °C for 30 s and annealing temperature for 1 min for 40 cycles with a 2.5 °C ramp rate, 98 °C for 1 min hold. For the UDP experiments, the extension time was increased to 1.5 min. Optimal annealing temperatures determined by gradient PCR were 57.6, 54 and 60 °C for *pfug* and *pfrct*, respectively. Droplets were read on a QX150 reader. Droplet populations were manually gated into negative, positive and double-positive populations using the QuantaSoft software.

Morphology of resistant mutants. The morphology of the resistant mutants was examined and imaged using Giemsa-stained slides under light microscopy (Nikon TS100 and DS-F2 camera) with ×100 magnification. The cultures were under drug pressure (38.4 nM GNF179).

Phenotypic reversion of mutations to WT. All 11 resistant mutants were cultured without drug pressure for 4 months. Phenotypic reversion was defined by a change in IC₅₀ values and morphology. IC₅₀ assays using the SYBR Green I drug sensitivity assay were carried out on the second and fourth month to evaluate changes in IC₅₀ values. Two-sided t-tests at α = 0.05 with unequal variances were performed to compare IC₅₀ at the second and fourth month. F-tests were performed to test for equality of variance (PIUGT: P = 0.778 > 0.05, PIAC-T: 0.03 > 0.05).

Localization of the UDP-galactose and the acetyl-CoA transporter. The full-length coding sequence for *pfug* (PF3D7_1113300) was amplified from genomic DNA using primers p4794/p4795 (Supplementary Table 3) and subcloned into a PD2-based expression vector downstream of the *P. berghei* EFalpha promoter and mRFP, yielding the expression plasmid PD2–PB-EF1–PfRFT–dHFR-BSD. This expression plasmid was then transferred into the T98G cell line together with the pINT plasmid to achieve attP × attB integrase-mediated recombination53. A plasmid (pDC2-GFP-Sec12–dHFR) expressing the ER marker, GFP–Sec12 was co-transfected with mRFP–PfRFT and pINT, and the parasites were selected with 2.5 μM WR99210/0.2 μg ml–1 blastcicidin/250 μg ml–1 G418 (ref. 24). In a similar manner to PfUGT, the full-length coding sequence for *PF3D7_1036800* was amplified from genomic DNA using primers indicated in Supplementary Table 3 and subcloned into a PD2-based expression vector downstream of the *P. falciparum* calmodulin promoter and downstream of GFp, yielding the expression plasmid pDC2–PICAMpro–PIAC-T–dHFR–BSD. This plasmid was transfected into the Dd2–atb parasite line together with the pINT plasmid to yield atpT × atbT integrase-mediated recombination and selected for as above53. The ER was stained with ER-tracker-red at 1 μg ml–1 for 15 min. Cultures were then washed twice with 1 × PBS and imaged.

Fluorescence microscopy imaging. Live-cell imaging of parasites expressing GFP- and mRFP-tagged proteins was performed on a Nikon TIE PFS inverted microscope equipped with a CoolSNAP HQ2 monochrome camera. Aliquots (100 μl) of parasite culture were washed once with RPMI 1640 medium lacking Albulmax II, transferred to MatTek poly–L-lysine-coated glass-bottom culture dishes, and overlaid with 1 ml of medium containing 2 μg ml–1 of the nuclear stain Hoechst 33342.

PIAC-T and PbACT homology modelling. Considering similarities in protein length (>500 residues) and sequence (33%), the MFS phosphate transporter from *P. falciparum* (PDB id 4J05) was used as the three-dimensional template alignment is shown in Supplementary Fig. 2. In addition, 846 sequences similar to PfACT were retrieved by BlastP and aligned with the fungi *Piriformospora indica* (PDB id 4J05) was used as the three-dimensional template to model PIAC-T and PbACT. The sequence alignment between PIAC-T and the template was calculated using ClustalW, followed by manually refining gaps based on the transmembrane structure and predicted using the TMpred server (http://www.ch.embnet.org). The alignment is shown in Supplementary Fig. 2. In addition, 846 sequences similar to PIAC-T were retrieved by BlastP and aligned
automatically with ClustalW and refined manually. The WebLogo server (http://weblogo.berkeley.edu/logo.cgi) was used to plot the amino acid distribution in a five-residue window around the mutated residue in PfACT and PbACT, as shown in Supplementary Fig. 3. The conservation score were also shown below each position. These scores were computed with the AL2CO program and were linearly rescaled from 0 (no conservation at this position) to 9 (highest conservation in the alignment). Three-dimensional models of PfACT and PbACT were constructed with MODELLER-9v16 (ref. 55). Among the 20 models generated for each of the proteins, the one with the lowest energy was selected as the final model.

Data availability. All evolved GNF179 and GNF156 resistant strains will be made available upon request to P.B. (early limiting dilution evolved Dd2 strains, including those used for all assays and all P. berghei strains) and/or E.A.W. (engineered Dd2 parasite lines and all GNF179-resistant strains obtained by previously reported methods). All relevant whole genome sequencing data have been uploaded to accessions (SRP062003 (P. berghei and early limiting dilution P. falciparum), SRP021001 (the higher dose ramp-up and high selection GNF179-resistant P. falciparum clones) and SRP075559 (additional P. berghei samples, which were treated with either 30 or 100 mg kg⁻¹ GNF179), as indicated in the Methods. All other data supporting the findings of this study are available within this Article and its Supplementary Information.

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**Author contributions**

M.Y.-X.L., G.L., M.C.S.L., E.A.W. and P.B. designed the experiments. M.Y.-X.L., G.L., M.C.S.L., C.R., B.H.T., V.C., B.F.T., A.C., M.N., B.M., E.D.C. and L.L. performed the experiments. Modelling work was performed by R.W. M.Y.-X.L., G.L., M.C.S.L., C.R., V.C., M.N., E.D.C. and P.G. analysed the data. G.M.C.B., P.C.-L.H., L.R., D.A.F. and T.T.D. contributed support. M.Y.-X.L., G.L., M.C.S.L., B.K.S.Y., D.A.F., E.A.W. and P.B. wrote and proofread the manuscript. E.A.W. and P.B. gave technical support and conceptual advice. The manuscript was edited by all authors.

**Additional information**

Supplementary information is available for this paper. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to E.A.W. and P.B.

**Competing interests**

The authors declare no competing financial interests.