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Ex vivo susceptibilities to ganaplacide and diversity in potential resistance mediators in Ugandan *Plasmodium falciparum* isolates

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ABSTRACT Novel antimalarials are urgently needed to combat rising resistance to available drugs. The imidazolopiperazine ganaplacide is a promising drug candidate, but decreased susceptibility of laboratory strains has been linked to polymorphisms in the *Plasmodium falciparum* cyclic amine resistance locus (PfCARL), acetyl-CoA transporter (PfACT), and UDP-galactose transporter (PfUGT). To characterize parasites causing disease in Africa, we assessed *ex vivo* drug susceptibilities to ganaplacide in 750 *P. falciparum* isolates collected in Uganda from 2017 to 2023. Drug susceptibilities were assessed using a 72-hour SYBR Green growth inhibition assay. The median IC_{50} for ganaplacide was 13.8 nM, but some isolates had up to 31-fold higher IC_{50}s (31/750 with IC_{50} > 100 nM). To assess genotype-phenotype associations, we sequenced genes potentially mediating altered ganaplacide susceptibility in the isolates using molecular inversion probe and dideoxy sequencing methods. PfCARL was highly polymorphic, with eight mutations present in >5% of isolates. None of these eight mutations had previously been selected in laboratory strains with *in vitro* drug pressure and none were found to be significantly associated with decreased ganaplacide susceptibility. Mutations in PfACT and PfUGT were found in ≤5% of isolates, except for two frequent (>20%) mutations in PfACT; one mutation in PfACT (I140V) was associated with a modest decrease in susceptibility. Overall, Ugandan *P. falciparum* isolates were mostly highly susceptible to ganaplacide. Known resistance mediators were polymorphic, but mutations previously selected with *in vitro* drug pressure were not seen, and mutations identified in the Ugandan isolates were generally not associated with decreased ganaplacide susceptibility.

KEYWORDS malaria, *Plasmodium falciparum*, ganaplacide, drug susceptibility, antimalarial drugs, PfCARL, PfACT, PfUGT

Malaria, in particular disease caused by *Plasmodium falciparum*, continues to cause hundreds of millions of illnesses and hundreds of thousands of deaths each year (1). Partial resistance to artemisinins, the most important drugs to treat falciparum malaria, is well-established in parts of Southeast Asia and has recently been described in eastern Africa (2–7). New antimalarials with novel mechanisms of action are urgently needed. Among the most advanced new antimalarial compounds under development is the imidazolopiperazine ganaplacide (KAF156) (8, 9).

Ganaplacide has demonstrated potent activity against cultured *P. falciparum,* with activity against liver (IC_{50} 5 nM), asexual erythrocytic (IC_{50} 6 nM), and sexual erythrocytic (IC_{50} 5 nM) life cycle stages (8, 10). Preclinical and clinical studies demonstrated activity against *P. falciparum* and *Plasmodium vivax* (11) and transmission-blocking potential (12). Ganaplacide has good oral bioavailability, favorable pharmacokinetics, and good safety and tolerability properties (13, 14). The compound has been tested in phase II

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clinical studies in combination with lumefantrine and determined to be well tolerated and efficacious as a 3-day treatment regimen (9). Phase III studies of ganaplacide-lumefantrine to treat falciparum malaria have been initiated. Ganaplacide is thus poised to be the first new non-artemisinin drug to treat falciparum malaria in decades.

The mechanism of action of ganaplacide is unknown, but parasites exposed to the drug *in vitro* developed mutations in the *P. falciparum* cyclic amine resistance locus (PfCARL), acetyl CoA transporter (PfACT), and UDP-galactose transporter (PfUGT) (15, 16). For PfCARL, 13 mutations were selected, mostly located near or in predicted transmembrane domains of the protein, and not in the catalytic site (16). Several of these mutations led to >40-fold increases in ganaplacide IC_{50}s, with up to µM drug tolerance in mutant parasites (8, 16). For PfACT, selected mutations included 20 single nucleotide polymorphisms, including stop mutations, some of which led to parasites with susceptibility only at µM levels (15). For PfUGT, one mutation was selected and associated with an about 500-fold shift in susceptibility to KAF179, a compound closely related to ganaplacide. The three proteins in which mutations were selected by ganaplacide or KAF179 are all predicted drug transporters located at the Golgi apparatus and endoplasmic reticulum (ER) (15, 16). Studies with *Saccharomyces cerevisiae* showed that imidazolopiperazine-resistant clones carried mutations in proteins involved in ER-based lipid homeostasis and autophagy. It was proposed that imidazolopiperazines might inhibit protein folding, sorting, and trafficking, thus interfering with the establishment of new permeation pathways and leading to ER expansion (16, 17). Several studies showed mediation of resistance to compounds from other chemical classes by mutations in PfCARL and PfACT, suggesting that mutations in these proteins alter drug transport, but that the proteins are not the biochemical targets of imidazolopiperazines (16, 18, 19).

To gain insights into potential resistance to imidazolopiperazines in parasites circulating in Africa, we tested susceptibilities to ganaplacide in fresh isolates causing malaria in eastern Uganda. We also characterized parasite genotypes, searched for genotype-phenotype associations, and considered associations between susceptibilities to ganaplacide and lumefantrine, its proposed partner drug.

RESULTS

Ex vivo susceptibilities to ganaplacide

We collected 750 fresh *P. falciparum* isolates from patients presenting with uncomplicated malaria between 2017 and 2023 in Tororo, Busia, and Mbale districts, all in eastern Uganda, and evaluated susceptibilities to ganaplacide. Half maximum inhibitory concentrations (IC_{50}s) were determined using a SYBR green assay. Ugandan parasites were mostly highly susceptible to ganaplacide, with a median IC_{50} of 13.8 nM (Fig. 1A). However, susceptibilities varied over a broad range (IC_{50} 1–426 nM), with IC_{50} > 100 nM for 31/750 (4.1%) of the isolates. For both ganaplacide and lumefantrine, susceptibilities of Ugandan isolates showed modest decreases in recent years, although results might have been affected by a slight loss of activity of stored compounds over time (Table S1).

Genotype-phenotype associations

Resistance of *P. falciparum* to ganaplacide has been linked to mutations selected *in vitro* in PfCARL, PfACT, and PfUGT. We characterized the genotypes of 1112 Ugandan isolates collected from 2015 to 2023, including 750 isolates for which ganaplacide IC_{50}s were determined. PfCARL was highly polymorphic amongst Ugandan isolates (116 mutations; Table S2). Considering the 3D7 strain as a reference, eight mutations were found in 5% or more of Ugandan isolates, with one mutation, K903E, detected in all studied isolates (Fig. 1B). None of these mutations have been previously shown to alter susceptibility to ganaplacide. Additionally, many parasites harbored duplications and deletions in PfCARL, especially in the regions spanning amino acids 245-246, 622-635, and 935-943. We identified 11 mutations in PfACT, of which 2 (I140V and T459I) were prevalent in over 20% of parasites (Fig. 1C). We identified four mutations in PfUGT, all in ≤5% of parasites

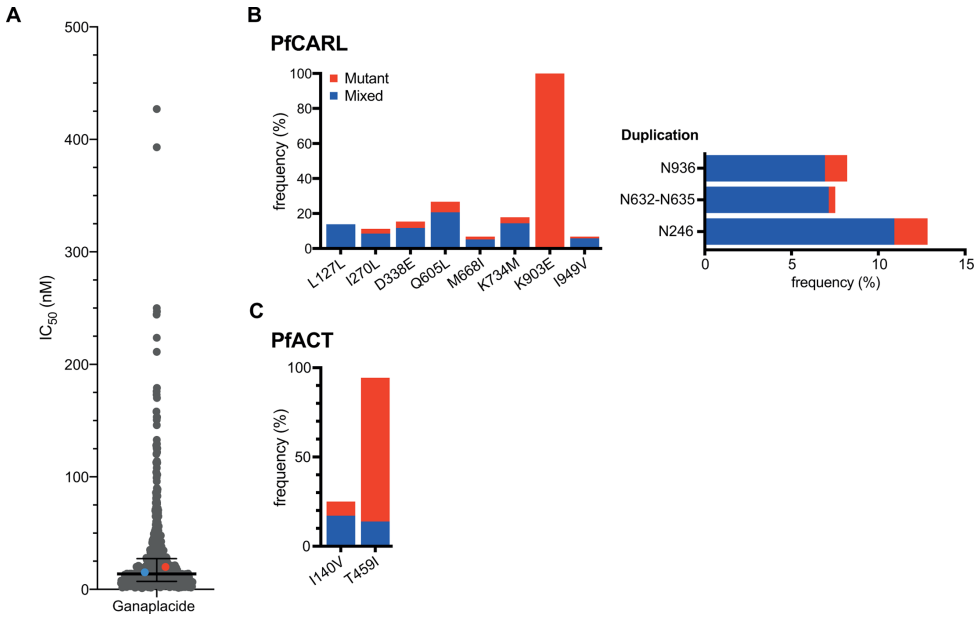

FIG 1 Susceptibilities to ganaplacide and mutations in resistance mediators of Ugandan parasites. (A) IC_{50}s of individual isolates (gray dots). The horizontal line shows the median and whiskers the interquartile range. Mean IC_{50}s of control parasites 3D7 (red) and Dd2 (blue) are depicted as colored dots. Mutations in PfCARL (B) and PfACT (C) were observed in >5% of Ugandan *P. falciparum* isolates. Depicted are mutation frequencies of mixed (WT and mutant; blue) and mutant (red) isolates.

(Table S2). Importantly, no mutations previously selected *in vitro* with ganaplacide pressure (8, 15, 16, 20) were seen in the Ugandan isolates. One PfCARL three codon duplication, N936dup, seen in 37 isolates, and one mutation, Q605L, seen in 26% of isolates, were associated with increases in ganaplacide susceptibility of borderline statistical significance (Table 1). A mutation in PfACT, P312S, was also associated with a significant increase in susceptibility. Isolates that carried the I140V mutation, seen in 25% of isolates, had a modest decrease in susceptibility compared to WT parasites (IC_{50} 12.5 nM for WT; 16.3 nM for mixed; 15.1 nM for mutant; WT vs mixed $P = 0.01$). Several isolates with mutations in the three transporter proteins had IC_{50}s shifts >twofold, but determining statistical significance was limited by the small sample size (Table S3). Overall, Ugandan *P. falciparum* isolates were highly polymorphic in PfCARL and PfACT, with fewer mutations in PfUGT, but only one mutation (PfACT I140V) was associated with significantly decreased susceptibility to ganaplacide compared to that of wild-type parasites.

Correlations between susceptibilities to ganaplacide and lumefantrine

Ganaplacide is under development in combination with lumefantrine as a new treatment for uncomplicated malaria. Although there is to date no evidence that ganaplacide and lumefantrine share resistance mechanisms, we assessed correlations between susceptibilities to the two compounds in Ugandan *ex vivo* isolates. We identified a weak positive correlation (r = 0.32) between the susceptibilities of individual isolates, with most isolates highly susceptible to both ganaplacide and lumefantrine (Fig. 2).

We more deeply explored the genotypes of the seven isolates with the highest ganaplacide IC_{50}s (Table 2). Isolates that displayed decreased susceptibility to ganaplacide were generally highly susceptible (IC_{50} < 10 nM) to lumefantrine, except for two isolates that had IC_{50}s of 211 nM and 224 nM for ganaplacide and 32.5 nM and 28.8 nM for lumefantrine, respectively. Interestingly, four out of the seven isolates with remarkably low ganaplacide susceptibility carried the PfCARL G605L mutation, which was present in 26.8% of all studied isolates. The seven isolates did not carry any PfK13 propeller domain mutations, which have recently been associated with artemisinin

TABLE 1 Genotype-phenotype associations

Compound	Mutation	IC_{50} (nM) (N[a])			*P*-value
		WT	Mixed	Mut	
Ganaplacide	PfCARL Q605L	12.6	15.0	8.5	0.03 (WT vs Mut)
		(365)	(105)	(28)	0.26 (WT vs Mixed)
	PfCARL N936dup[b]	13.3	9.4	7.9	0.05 (WT vs Mut)
		(387)	(30)	(7)	0.42 (WT vs Mixed)
	PfACT I140V	12.5	16.3	15.1	0.16 (WT vs Mut)
		(420)	(103)	(46)	0.01 (WT vs Mixed)
	PfACT P312S	13.4	2.7	NA[c]	0.03 (WT vs Mixed)
		(566)	(2)		
	PfK13 C469Y	12.7	23.6	74.8	0.18 (WT vs Mixed)
		(491)	(5)	(1)	
	PfK13 A675V	12.4	57.1	6.9	0.59 (WT vs Mut)
		(486)	(3)	(2)	0.02 (WT vs Mixed)
	PfK13 A578S	12.7	9.7	5.3	0.05 (WT vs Mut)
		(473)	(8)	(5)	0.50 (WT vs Mixed)

[a]Number of isolates with the indicated mutation considered for this analysis.
[b]Dup, duplication.
[c]NA, not available (no fully mutant samples were collected).

partial resistance in Uganda (5, 6), and only one out of these seven isolates carried the *P. falciparum* chloroquine resistance transporter (PfCRT) K76T mutation that mediates chloroquine resistance. All parasites carried five dihydrofolate reductase (PfDHFR) N51I/C59R/S108N and dihydropteroate synthetase (PfDHPS) A437G/L540E mutations, and five out of seven carried the multidrug resistance protein 1 (PfMDR1) Y184F mutation,

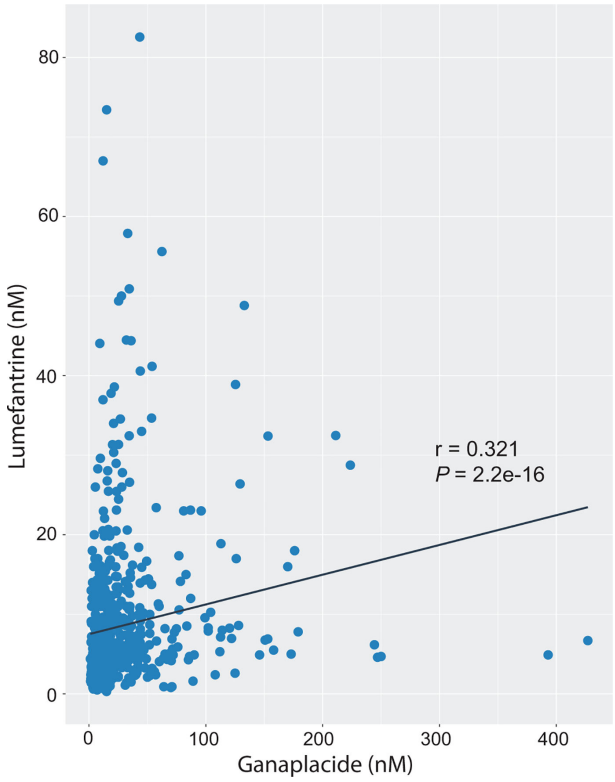

FIG 2 Spearman rank correlation between susceptibilities of Ugandan parasites to lumefantrine and ganaplacide. Blue dots depict results for single isolates. The black line demonstrates the linear regression.

TABLE 2 Susceptibilities of and mutations in *P. falciparum* isolates with unusually high ganaplacide IC_{50}s

	IC_{50} (nM)		Mutations[a]					
Parasite	Ganaplacide	Lumefantrine	PfCARL	PfK13	PfCRT	PfMDR1	PfDHFR	PfDHPS
MAS-172	427	6.7	G605L, N631Y	H136N, L189T, T49S	–	N6621del, D650-N660del, Y184F	N51I, C59R, S108N	A437G, K540E, S436H
MAS-168	393	4.9	G605L, N206S, N246dup, D226N, I949V, L207I, K1150N, K734M, Y366H	N142del, L189T	R371I, N75G, D24Y, L76T, M74I, S14N	N659-N661del, N660-N661del, N661del, N661dup, D650-N657del, D650-N658del, D650-N660del, G968A, Y184F	N51I, C59R, S108N, I164L	A437G, K540E, S436H
MAS-240	250	4.7	G605L	L258M	–	N658-N661del, N659-N661del, N6621dup, Y184F	N51I, C59R, S108N	A437G, K540E
MAS-169	247	4.6	–	N142del	–	D1246Y, Y184F	N51I, C59R, S108N	A437G, K540E
PBC-548_09Nov22	244	6.2	NA	NA	NA	NA	NA	NA
PBC-685_09Nov22	224	28.8	–	–	–	–	N51I, C59R (S108N:NA)	A437G, K540E
PBC-308	211	32.5	G605L	–	–	Y184F	N51I, C59R, S108N	A437G, K540E

[a]Del, deletion; dup, duplication; NA, not available; –, no mutation.

consistent with expected prevalences in Uganda (5). Overall, susceptibility results for ganaplacide and lumefantrine showed a modest positive correlation and genotype-phenotype analysis did not reveal a significant association between the isolates with the highest ganaplacide IC_{50}s and specific mutations in PfCARL or other resistance markers.

Susceptibilities to ganaplacide in isolates with artemisinin resistance mediating PfK13 mutations

The C469Y and A675V PfK13 mutations, which are validated markers of artemisinin partial resistance, have emerged in northern Uganda (5, 6). We tested whether these mutations were associated with altered susceptibilities to ganaplacide. Isolates harboring either the C469Y (four isolates; median IC_{50} 18.2 nM; WT vs mutant $P = 0.38$) or A675V (three isolates; 22.9 nM; $P = 0.72$) mutation had lower susceptibilities than those for isolates with wild-type K13 sequences (12.0 nM; Fig. 3). Of note, two isolates that had both mutations, probably present in different parasites within a mixed isolate, had much lower susceptibility to ganaplacide (70.6 nM; $P = 0.07$), although the small numbers of mutant isolates limited this analysis. The PfK13 A578S mutation, which has been identified across Africa at low prevalence but has not been linked to artemisinin partial resistance, was associated with increased ganaplacide susceptibility (five isolates; median IC_{50} 5.3 nM; WT vs mutant $P = 0.05$; Table 1).

DISCUSSION

The recent emergence of partial resistance to artemisinins in Africa underscores the urgent need for new antimalarials with novel modes of action (2–7). Ganaplacide is a promising drug candidate that does not appear to share mechanisms of action or resistance with available antimalarials. However, resistance to ganaplacide has been selected *in vitro*, and it is important to determine if parasites now circulating in Uganda are uniformly susceptible to the compound. We therefore studied the susceptibilities of

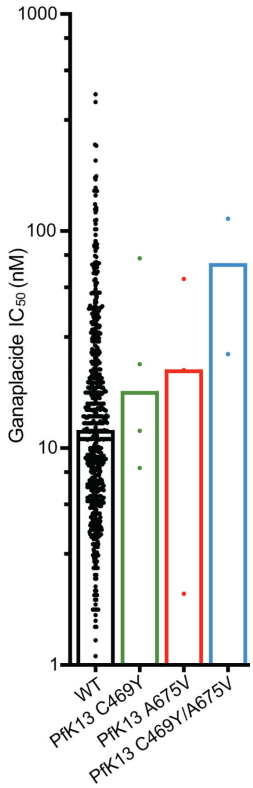

FIG 3 Ganaplacide susceptibilities of Ugandan *P. falciparum* isolates with validated K13 mutations. Dots depict single isolates and bar graphs the median IC_{50}s.

fresh isolates from eastern Uganda. Isolates were mostly highly susceptible to ganaplacide. Although known resistance mediators were polymorphic, previously selected resistance mutations were not seen, and only four polymorphisms identified in PfACT and PfCARL in Ugandan isolates were modestly associated with altered susceptibility to ganaplacide.

Our analysis of 750 Ugandan isolates showed mostly excellent activity of ganaplacide, with a median IC_{50} of 13.8 nM, similar to that seen with laboratory strains of *P. falciparum* (8). However, we observed variability in activities, with up to 31-fold differences in IC_{50}s between isolates. This raised the question of whether mutations in PfCARL, PfACT, or PfUGT, the proteins shown to mediate resistance *in vitro*, drove differences in susceptibility. Analysis of these three predicted transporters in Ugandan isolates showed that PfCARL was highly polymorphic, with lower numbers of polymorphisms in PfACT and PfUGT. Importantly, none of the mutations identified in the field isolates were those previously selected by *in vitro* drug pressure or reported to be associated with altered drug susceptibility. Two polymorphisms in PfCARL were significantly associated with increased susceptibility to ganaplacide. One of these, the N936 duplication, was in a low complexity region with many asparagine repeats and the other was a Q605L mutation. Curiously, the Q605L mutation was also seen in multiple isolates with unusually low susceptibilities, so true associations with ganaplacide susceptibility are uncertain. Several mutations in PfCARL that were seen in isolates with lower ganaplacide susceptibility were located in the predicted VHS (Vsp-27, Hrs, and STAM) domain of PfCARL, which potentially plays a role in cargo recognition and trans-Golgi trafficking (16, 21). This result suggests a potential role for these mutations in mediating altered ganaplacide activity, but the few mutant samples available for study limited our ability to characterize the impact of these mutations. In contrast, ganaplacide-resistance conferring mutations selected previously *in vitro* were mostly in or near the seven transmembrane regions (located within bp 790–1,283) of PfCARL (16). We identified one mutation, L1142I, in

the fifth transmembrane domain of PfCARL in the Ugandan isolates, but this mutation was at low prevalence, only found in mixed isolates, and not associated with ganaplacide activity (Table S2). The PfACT I140V mutation, which was associated with a significant, albeit modest decrease in ganaplacide susceptibility, is located near the fourth transmembrane domain of the protein. Most PfACT mutations selected *in vitro* by drug pressure and found to be associated with decreased susceptibility to ganaplacide were located in or near transmembrane domains, potentially indicating that the PfACT I140V mutation seen in Ugandan isolates could play a role in ganaplacide susceptibility. It will be of interest to introduce naturally occurring PfCARL and PfACT mutations into *P. falciparum* strains to determine if they lead to altered ganaplacide activity and/or parasite fitness.

A phase II clinical trial confirmed the safety, tolerability, and efficacy of ganaplacide plus lumefantrine in the treatment of uncomplicated *P. falciparum* malaria (9). We identified a slight positive correlation between susceptibilities to these two drugs. Although there is no evidence for shared resistance mechanisms between ganaplacide and lumefantrine, these results suggest the need for continued study to identify mechanisms that might mediate decreased susceptibility to both compounds. This is relevant, as modest decreases in susceptibility to lumefantrine have been identified among Ugandan *P. falciparum* isolates collected in recent years (22, 23), and treatment failures have been noted when activities of both components of a combination are compromised, e.g., with sulfadoxine-pyrimethamine or dihydroartemisinin-piperaquine (24, 25).

With the recent emergence of artemisinin partial resistance in eastern Africa (3–6, 26), it is important to determine if PfK13 mutations that mediate resistance are associated with altered activities of other drugs. Indeed, the PfK13 C469Y mutation was strongly associated with decreased lumefantrine susceptibility in Ugandan isolates (23). We compared the susceptibilities of isolates with PfK13 C469Y and A675V mutations to those of isolates with PfK13 WT alleles at these loci. The two PfK13 mutations were associated with modest decreases in the activity of ganaplacide, but the small number of samples available for study limited our analysis. With the predicted spread of multiple PfK13 mutations that mediate artemisinin partial resistance it will be important to continue to assess the potencies and clinical activities of new antimalarial compounds, such as ganaplacide, against *P. falciparum* isolates with a range of genotypes.

Our study had limitations. Most importantly, testing *ex vivo* susceptibilities of fresh clinical isolates necessarily only allows for one measurement. Potentially spurious findings could not be confirmed with a repeat assay, and some unusual results (e.g., ganaplacide IC_{50}s > 200 nM) may have been due to limited compound solubility or other factors not indicative of true drug resistance. Second, control strains were tested monthly and not with every assay, further limiting our ability to identify spurious readings. Third, the frequencies of some studied genetic polymorphisms were low, limiting the power to detect meaningful associations despite studying a large number of samples. Despite these concerns, our testing of a large number of samples over a long period offers assurance that our composite results accurately represent the ganaplacide susceptibilities of Ugandan clinical isolates.

Ganaplacide is a promising antimalarial drug candidate, and ganaplacide-lumefantrine is entering phase III trials for the treatment of uncomplicated malaria. Studies of ganaplacide susceptibilities and the genetic diversity of resistance markers in parasites circulating in Africa are vital to inform drug development strategies. Our data show that *ex vivo* susceptibility to ganaplacide was mostly excellent in Ugandan *P. falciparum* isolates. Some isolates with reduced susceptibility were observed, but altered susceptibility was not explained by differences in known parasite resistance markers. Our results support the development of ganaplacide as a new drug to treat malaria but also continued surveillance for potential selection of resistance during phase III trials and subsequent deployment as a new antimalarial agent.

MATERIALS AND METHODS

Samples for study

Subjects over 6 months of age presenting between December 2015 and April 2023 at three outpatient clinics in eastern Uganda (Tororo District Hospital, Tororo District; Masafu General Hospital, Busia District; or Busiu Health Center, Mbale District) or enrolled in cohort studies in this area with clinical suspicion for malaria and a positive Giemsa-stained blood film for *P. falciparum* and without signs of severe disease were enrolled after informed consent, as previously described (27). About 12 isolates were collected and studied each month. Blood was collected in a heparinized tube after which, following national guidelines, patients were treated with artemether-lumefantrine.

Ex vivo drug susceptibility assays

A total of 750 samples, collected from 2017 to 2023, were processed and drug susceptibility assays were performed as previously described (22, 27, 28). In brief, samples with parasitemias ≥0.3% were placed into the culture after the removal of plasma and buffy coat. Aliquots of blood samples were stored on Whatman 3 MM filter paper or placed into RNAlater (Invitrogen) and stored at −20°C. Ganaplacide and lumefantrine, provided by Medicines for Malaria Venture, were prepared as 10 mM stock solutions in dimethyl sulfoxide and stored at −20°C.

For susceptibility assays, drugs were serially diluted threefold (range 1 uM to 0.05 nM for ganaplacide and lumefantrine) in complete media and parasites were added (parasitemia 0.2% and hematocrit 2%). Drug-free and parasite-free controls were included. Plates were incubated for 72 hours in a humidified modular incubator under 90% N_2, 5% CO_2, and 5% O_2 at 37°C. Parasite growth was quantified based on SYBR Green fluorescence (485 nm excitation/530 nm emission). Dd2 and 3D7 laboratory control strains (BEI Resources) were assessed monthly. IC_{50}s were derived and variability in results was assessed as previously described (27).

Sequencing of Ugandan *P. falciparum* DNA

DNA from dried blood spots was extracted using Chelex-100. RNA was extracted from samples stored in RNAlater using the PureLink RNA Mini Kit (Invitrogen) before treatment with DNAseI (NEB). cDNA was reverse transcribed using the SuperScript IV First-Stand Synthesis kit (Invitrogen) according to the manufacturer's protocol using hexamer primers.

For all samples, sequences of *pfcarl* and *pfact* were determined using molecular inversion probe (MIP) capture and deep sequencing (5, 28, 29). Experimental design, probes, and primers (Table S5) were used as described previously (5, 28, 29).

Dideoxy sequencing was performed for intron-rich regions that did not yield good quality sequences by MIP analysis when either gDNA (for *pfugt*) or RNA (for *pfcarl* and *pfact*) was available. The genes encoding PfACT, PfUGT, and the c-terminal region of PfCARL were amplified by PCR using sequence-specific primers (Table S4). PCR products were purified with Ampure beads (Beckman Coulter) and sequenced by dideoxy techniques. Sequences were analyzed with CodonCodeAligner (CodonCode Corporation) based on the 3D7 reference sequence.

Statistical analyses

Associations between genotypes and drug susceptibilities were analyzed with the Wilcoxon test using R. Associations between IC_{50} values for lumefantrine and ganaplacide were assessed with the Spearman's rank test using R studio. The direction and magnitude of associations between IC_{50} values were quantified with Spearman's rank correlation coefficient (P = 2.2e−16). The statistical tests were two-tailed and considered significant at $P \leq 0.05$. Drug susceptibility trends over time were analyzed with the Mann-Kendall package in R.

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Medicines for Malaria Venture (MMV)	RD/15/0001	Roland A. Cooper

DATA AVAILABILITY

Raw sequencing reads for target genes are available in the NCBI Sequence Read Archive under accession numbers PRJNA660547, PRJNA850445, PP536578-PP536634, PP536673-PP537115, PP534096-PP534160, and PP533542-PP533545. MIP probes and PCR primers used in this study are listed in Tables S4 and S5 or prior publications (5, 23). MIPWrangler (https://github.com/bailey-lab/MIPWrangler), and MIPTools (https://github.com/bailey-lab/MIPTools) software are available on GitHub. Additional data and stored isolates are available from the authors upon request.

ETHICS APPROVAL

Study approval was obtained from the Makerere University Research and Ethics Committee, the Uganda National Council for Science and Technology, and the University of California, San Francisco, Committee on Human Research.

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental tables (AAC00466-24-S0001.docx). Tables S1 to S5.

REFERENCES

1. World Health Organization. 2023. World malaria report 2023. Geneva World Health Organization
2. Dondorp AM, Nosten F, Yi P, Das D, Phyo AP, Tarning J, Lwin KM, Ariey F, Hanpithakpong W, Lee SJ, Ringwald P, Silamut K, Imwong M, Chotivanich K, Lim P, Herdman T, An SS, Yeung S, Singhasivanon P, Day NPJ, Lindegardh N, Socheat D, White NJ. 2009. Artemisinin resistance in *Plasmodium falciparum* malaria. N Engl J Med 361:455–467. https://doi.org/10.1056/NEJMoa0808859
3. Uwimana A, Umulisa N, Venkatesan M, Svigel SS, Zhou Z, Munyaneza T, Habimana RM, Rucogoza A, Moriarty LF, Sandford R, et al. 2021. Association of *Plasmodium falciparum* kelch13 R561H genotypes with delayed parasite clearance in Rwanda: an open-label, single-arm, multicentre, therapeutic efficacy study. Lancet Infect Dis 21:1120–1128. https://doi.org/10.1016/S1473-3099(21)00142-0
4. Balikagala B, Fukuda N, Ikeda M, Katuro OT, Tachibana SI, Yamauchi M, Opio W, Emoto S, Anywar DA, Kimura E, Palacpac NMQ, Odongo-Aginya EI, Ogwang M, Horii T, Mita T. 2021. Evidence of artemisinin-resistant malaria in Africa. N Engl J Med 385:1163–1171. https://doi.org/10.1056/NEJMoa2101746
5. Asua V, Conrad MD, Aydemir O, Duvalsaint M, Legac J, Duarte E, Tumwebaze P, Chin DM, Cooper RA, Yeka A, Kamya MR, Dorsey G, Nsobya SL, Bailey J, Rosenthal PJ. 2021. Changing prevalence of potential mediators of aminoquinoline, antifolate, and artemisinin resistance across Uganda. J Infect Dis 223:985–994. https://doi.org/10.1093/infdis/jiaa687
6. Conrad MD, Asua V, Garg S, Giesbrecht D, Niaré K, Smith S, Namuganga JF, Katairo T, Legac J, Crudale RM, Tumwebaze PK, Nsobya SL, Cooper RA, Kamya MR, Dorsey G, Bailey JA, Rosenthal PJ. 2023. Evolution of partial resistance to artemisinins in malaria parasites in Uganda. N Engl J Med 389:722–732. https://doi.org/10.1056/NEJMoa2211803
7. Mihreteab S, Platon L, Berhane A, Stokes BH, Warsame M, Campagne P, Criscuolo A, Ma L, Petiot N, Doderer-Lang C, Legrand E, Ward KE, Zehaie Kassahun A, Ringwald P, Fidock DA, Ménard D. 2023. Increasing prevalence of artemisinin-resistant HRP2-negative malaria in eritrea. N Engl J Med 389:1191–1202. https://doi.org/10.1056/NEJMoa2210956
8. Kuhen KL, Chatterjee AK, Rottmann M, Gagaring K, Borboa R, Buenviaje J, Chen Z, Francek C, Wu T, Nagle A, et al. 2014. KAF156 is an antimalarial clinical candidate with potential for use in prophylaxis, treatment, and prevention of disease transmission. Antimicrob Agents Chemother 58:5060–5067. https://doi.org/10.1128/AAC.02727-13
9. Ogutu B, Yeka A, Kusemererwa S, Thompson R, Tinto H, Toure AO, Uthaisin C, Verma A, Kibuuka A, Lingani M, et al. 2023. Ganaplacide (KAF156) plus lumefantrine solid dispersion formulation combination for uncomplicated *Plasmodium falciparum* malaria: an open-label, multicentre, parallel-group, randomised, controlled, phase 2 trial. Lancet Infect Dis 23:1051–1061. https://doi.org/10.1016/S1473-3099(23)00209-8
10. Nagle A, Wu T, Kuhen K, Gagaring K, Borboa R, Francek C, Chen Z, Plouffe D, Lin X, Caldwell C, et al. 2012. Imidazolopiperazines: lead optimization of the second-generation antimalarial agents. J Med Chem 55:4244–4273. https://doi.org/10.1021/jm300041e
11. White NJ, Duong TT, Uthaisin C, Nosten F, Phyo AP, Hanboonkunupakarn B, Pukrittayakamee S, Jittamala P, Chuthasmit K, Cheung MS, Feng Y, Li R, Magnusson B, Sultan M, Wieser D, Xun X, Zhao R, Diagana TT, Pertel P, Leong FJ. 2016. Antimalarial activity of KAF156 in *Falciparum* and vivax malaria. N Engl J Med 375:1152–1160. https://doi.org/10.1056/NEJMoa1602250
12. Yipsirimetee A, Chiewpoo P, Tripura R, Lek D, Day NPJ, Dondorp AM, Pukrittayakamee S, White NJ, Chotivanich K. 2022. Assessment *in vitro* of the antimalarial and transmission-blocking activities of cipargamin and ganaplacide in artemisinin-resistant *Plasmodium falciparum*. Antimicrob Agents Chemother 66:e0148121. https://doi.org/10.1128/aac.01481-21
13. Leong FJ, Zhao R, Zeng S, Magnusson B, Diagana TT, Pertel P. 2014. A first-in-human randomized, double-blind, placebo-controlled, single- and multiple-ascending oral dose study of novel imidazolopiperazine KAF156 to assess its safety, tolerability, and pharmacokinetics in healthy adult volunteers. Antimicrob Agents Chemother 58:6437–6443. https://doi.org/10.1128/AAC.03478-14
14. Kublin JG, Murphy SC, Maenza J, Seilie AM, Jain JP, Berger D, Spera D, Zhao R, Soon RL, Czartoski JL, Potochnic MA, Duke E, Chang M, Vaughan A, Kappe SHI, Leong FJ, Pertel P, Prince WT, KAF156 Study Team. 2021. Safety, pharmacokinetics, and causal prophylactic efficacy of KAF156 in a *Plasmodium falciparum* human infection study. Clin Infect Dis 73:e2407–e2414. https://doi.org/10.1093/cid/ciaa952
15. Lim MY-X, LaMonte G, Lee MCS, Reimer C, Tan BH, Corey V, Tjahjadi BF, Chua A, Nachon M, Wintjens R, Gedeck P, Malleret B, Renia L, Bonamy GMC, Ho PC-L, Yeung BKS, Chow ED, Lim L, Fidock DA, Diagana TT, Winzeler EA, Bifani P. 2016. UDP-galactose and acetyl-CoA transporters as *Plasmodium* multidrug resistance genes. Nat Microbiol 1:16166. https://doi.org/10.1038/nmicrobiol.2016.166
16. LaMonte G, Lim MY-X, Wree M, Reimer C, Nachon M, Corey V, Gedeck P, Plouffe D, Du A, Figueroa N, Yeung B, Bifani P, Winzeler EA. 2016. Mutations in the *Plasmodium falciparum* cyclic amine resistance locus (PfCARL) confer multidrug resistance. mBio 7:e00696-16. https://doi.org/10.1128/mBio.00696-16
17. LaMonte GM, Rocamora F, Marapana DS, Gnädig NF, Ottilie S, Luth MR, Worgall TS, Goldgof GM, Mohunlal R, Santha Kumar TR, et al. 2020. Pan-active imidazolopiperazine antimalarials target the *Plasmodium falciparum* intracellular secretory pathway. Nat Commun 11:1780. https://doi.org/10.1038/s41467-020-15440-4
18. Imlay LS, Lawong AK, Gahalawat S, Kumar A, Xing C, Mittal N, Wittlin S, Churchyard A, Niederstrasser H, Crespo-Fernandez B, Posner BA, Gamo F-J, Baum J, Winzeler EA, Laleu B, Ready JM, Phillips MA. 2023. Fast-killing tyrosine amide ((*S*)-SW228703) with blood- and liver-stage antimalarial activity associated with the cyclic amine resistance locus (*Pf*CARL). ACS Infect Dis 9:527–539. https://doi.org/10.1021/acsinfecdis.2c00527
19. Magistrado PA, Corey VC, Lukens AK, LaMonte G, Sasaki E, Meister S, Wree M, Winzeler E, Wirth DF. 2016. *Plasmodium falciparum* cyclic amine resistance locus (PfCARL), a resistance mechanism for two distinct compound classes. ACS Infect Dis 2:816–826. https://doi.org/10.1021/acsinfecdis.6b00025
20. Meister S, Plouffe DM, Kuhen KL, Bonamy GMC, Wu T, Barnes SW, Bopp SE, Borboa R, Bright AT, Che J, et al. 2011. Imaging of *Plasmodium* liver stages to drive next-generation antimalarial drug discovery. Science 334:1372–1377. https://doi.org/10.1126/science.1211936

21. Wang T, Liu NS, Seet LF, Hong W. 2010. The emerging role of VHS domain-containing Tom1, Tom1L1 and Tom1L2 in membrane trafficking. Traffic 11:1119–1128. https://doi.org/10.1111/j.1600-0854.2010.01098.x
22. Tumwebaze P.K, Katairo T, Okitwi M, Byaruhanga O, Orena S, Asua V, Duvalsaint M, Legac J, Chelebieva S, Ceja FG, Rasmussen SA, Conrad MD, Nsobya SL, Aydemir O, Bailey JA, Bayles BR, Rosenthal PJ, Cooper RA. 2021. Drug susceptibility of *Plasmodium falciparum* in eastern Uganda: a longitudinal phenotypic and genotypic study. Lancet Microbe 2:e441–e449. https://doi.org/10.1016/s2666-5247(21)00085-9
23. Tumwebaze Patrick K., Conrad MD, Okitwi M, Orena S, Byaruhanga O, Katairo T, Legac J, Garg S, Giesbrecht D, Smith SR, Ceja FG, Nsobya SL, Bailey JA, Cooper RA, Rosenthal PJ. 2022. Decreased susceptibility of *Plasmodium falciparum* to both dihydroartemisinin and lumefantrine in northern Uganda. Nat Commun 13:6353. https://doi.org/10.1038/s41467-022-33873-x
24. Amaratunga C, Lim P, Suon S, Sreng S, Mao S, Sopha C, Sam B, Dek D, Try V, Amato R, Blessborn D, Song L, Tullo GS, Fay MP, Anderson JM, Tarning J, Fairhurst RM. 2016. Dihydroartemisinin-piperaquine resistance in *Plasmodium falciparum* malaria in Cambodia: a multisite prospective cohort study. Lancet Infect Dis 16:357–365. https://doi.org/10.1016/S1473-3099(15)00487-9
25. Plowe CV. 2022. Malaria chemoprevention and drug resistance: a review of the literature and policy implications. Malar J 21:104. https://doi.org/10.1186/s12936-022-04115-8
26. Uwimana A, Legrand E, Stokes BH, Ndikumana J-L, Warsame M, Umulisa N, Ngamije D, Munyaneza T, Mazarati J-B, Munguti K, Campagne P, Criscuolo A, Ariey F, Murindahabi M, Ringwald P, Fidock DA, Mbituyumuremyi A, Menard D. 2020. Emergence and clonal expansion of *in vitro* artemisinin-resistant *Plasmodium falciparum* kelch13 R561H mutant parasites in Rwanda. N Med 26:1602–1608. https://doi.org/%2010.1038/s41591-020-1005-2
27. Rasmussen SA, Ceja FG, Conrad MD, Tumwebaze PK, Byaruhanga O, Katairo T, Nsobya SL, Rosenthal PJ, Cooper RA. 2017. Changing antimalarial drug sensitivities in Uganda. Antimicrob Agents Chemother 61:e01516-17. https://doi.org/10.1128/AAC.01516-17
28. Kreutzfeld O, Tumwebaze PK, Okitwi M, Orena S, Byaruhanga O, Katairo T, Conrad MD, Rasmussen SA, Legac J, Aydemir O, Giesbrecht D, Forte B, Campbell P, Smith A, Kano H, Nsobya SL, Blasco B, Duffey M, Bailey JA, Cooper RA, Rosenthal PJ. 2023. Susceptibility of Ugandan *Plasmodium falciparum* isolates to the antimalarial drug pipeline. Microbiol Spectr 11:e0523622. https://doi.org/10.1128/spectrum.05236-22
29. Aydemir O, Janko M, Hathaway NJ, Verity R, Mwandagalirwa MK, Tshefu AK, Tessema SK, Marsh PW, Tran A, Reimonn T, Ghani AC, Ghansah A, Juliano JJ, Greenhouse BR, Emch M, Meshnick SR, Bailey JA. 2018. Drug-resistance and population structure of *Plasmodium falciparum* across the democratic republic of congo using high-throughput molecular inversion probes. J Infect Dis 218:946–955. https://doi.org/10.1093/infdis/jiy223