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Plasmodium malariae and *Plasmodium falciparum* comparative susceptibility to antimalarial drugs in Mali

Laurent Dembele [1]*†, Yaw Aniweh[2,3]†, Nouhoum Diallo[1], Fanta Sogore[1], Cheick Papa Oumar Sangare[1], Aboubecrin Sedhigh Haidara[1], Aliou Traore[1], Seidina A. S. Diakité[1,2], Mahamadou Diakite[1], Brice Campo[4], Gordon A. Awandare[2,3] and Abdoulaye A. Djimde[1]

[1]Malaria Research and Training Centre (MRTC), Faculty of Pharmacy, Université des Sciences, des Techniques et des Technologies de Bamako (USTTB); Point G, P.O. Box: 1805, Bamako, Mali; [2]West African Centre for Cell Biology of Infectious Pathogens (WACCBIP), Volta Road, Legon, Accra, Ghana; [3]Department of Biochemistry, Cell and Molecular Biology, College of Basic and Applied Sciences, University of Ghana, Legon, Ghana; [4]Medicines for Malaria Venture (MMV) ICC Building Entrance G, 3rd floor Route de Pré-Bois 20 Post Box 1826 CH-1215, Geneva 15, Switzerland

*Corresponding author. E-mail: laurent@icermali.org
†Contributed equally to this work.

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Objectives: To evaluate *Plasmodium malariae* susceptibility to current and lead candidate antimalarial drugs.

Methods: We conducted cross-sectional screening and detection of all *Plasmodium* species malaria cases, which were nested within a longitudinal prospective study, and an *ex vivo* assessment of efficacy of a panel of antimalarials against *P. malariae* and *Plasmodium falciparum,* both PCR-confirmed mono-infections. Reference compounds tested included chloroquine, lumefantrine, artemether and piperaquine, while candidate antimalarials included the imidazolopiperazine GNF179, a close analogue of KAF156, and the *Plasmodium* phosphatidylinositol-4-OH kinase (PI4K)-specific inhibitor KDU691.

Results: We report a high frequency (3%–15%) of *P. malariae* infections with a significant reduction in *ex vivo* susceptibility to chloroquine, lumefantrine and artemether, which are the current frontline drugs against *P. malariae* infections. Unlike these compounds, potent inhibition of *P. malariae* and *P. falciparum* was observed with piperaquine exposure. Furthermore, we evaluated advanced lead antimalarial compounds. In this regard, we identified strong inhibition of *P. malariae* using GNF179, a close analogue of KAF156 imidazolopiperazines, which is a novel class of antimalarial drug currently in clinical Phase IIb testing. Finally, in addition to GNF179, we demonstrated that the *Plasmodium* PI4K-specific inhibitor KDU691 is highly inhibitory against *P. malariae* and *P. falciparum*.

Conclusions: Our data indicated that chloroquine, lumefantrine and artemether may not be suitable for the treatment of *P. malariae* infections and the potential of piperaquine, as well as new antimalarials imidazolopiperazines and PI4K-specific inhibitor, for *P. malariae* cure.

Introduction

Worldwide malaria cases are estimated at 228 million with 93% cases occurring in sub-Saharan Africa. Malaria accounted for approximately 405 000 deaths globally in 2018.[1] Global efforts toward malaria elimination would be futile if treatments of the neglected non-*falciparum Plasmodium* species, such as *Plasmodium malariae*, are ineffective. *P. malariae* is widespread in sub-Saharan Africa[2–4] and the southeast area of the Pacific region where its prevalence has surpassed 30%.[5] Like *Plasmodium falciparum, P. malariae* infection is also reported to be associated with a high burden of anaemia[6] and can result in chronic infection if not well treated. *P. malariae* infections can also cause a high burden of morbidity associated with severe illness and possibly death.[7] Therefore, the clinical impacts of *P. malariae* malaria require that it be given a priority in the context of disease-elimination strategies, e.g. as reported for *Plasmodium vivax*.[8] A recent report from Victor Yman[2] indicated a decline of *P. falciparum* infection in 2016 yet a 2- to 6-fold increase of *P. malariae* and *Plasmodium ovale* spp. infections, respectively, when compared with 2010. Thus, it is important to tackle *P. malariae* in order to reach the elimination milestone.

Conventional microscopy is the gold standard for diagnosing malaria before the initiation of antimalarial therapy according to

the WHO.[1] However, use of microscopy is heavily marred by human errors causing misdiagnosis of *P. malariae*[9] while rapid diagnostic tests are only available for *P. falciparum* and *P. vivax* detection.[10,11] PCR assays can be used to detect the different *Plasmodium* species; however, they are time consuming and not readily applicable in a routine diagnostic setting at the health-facility level. In addition to the challenges with diagnostics, little is known about the efficacy of current artemisinin-based combination therapies (ACTs) against *P. malariae*. While ACTs are highly efficacious in treating *P. falciparum* malaria in Africa,[12,13] increasing failures using artemether/lumefantrine against *P. malariae* have been reported.[14] The WHO recommends ACTs for the treatment of *P. falciparum* malaria and primaquine, tafenoquine and ACTs or chloroquine (depending on the 8-aminoquinoline) for *P. vivax* and *P. ovale* malaria, yet there are no guidelines for the treatment of *P. malariae*. Because of no widespread evidence of chloroquine clinical resistance in *P. malariae*, the US CDC suggests chloroquine for *P. malariae* infections; however, this is no longer used in most sub-Saharan African countries. As the second most abundant malaria-causing parasite in the sub-Saharan region, there is the need to identify compounds that are effective against *P. malariae*. Indeed, the *P. malariae* parasite reservoir represents a hurdle to achieve the malaria elimination goal. This study aimed to evaluate *P. malariae* susceptibility to current and lead candidate antimalarial drugs.

Materials and methods

Study design, site and population screen

We conducted cross-sectional screening and detection of all *Plasmodium* species malaria cases, which were nested within a longitudinal prospective study, and an *ex vivo* assessment of efficacy of a panel of antimalarials against *P. malariae* and *P. falciparum* as schematized in Figure 1. This study was conducted from January to December 2019 in Faladje, a rural village located in the Koulikoro region, 80 km from Bamako. Malaria is hyper-endemic and highly seasonal in Faladje.[15] Children older than 1 year and non-pregnant adults with uncomplicated or asymptomatic malaria were enrolled if written informed consent was obtained from patients, parents, or guardians of children. Patients with severe malaria, chronic HIV or TB infection, or have taken an antimalarial drug during the past 14 days were excluded.

After a census of the site community, study subjects ($n = 4000$) were screened for inclusion and non-inclusion criteria after obtaining community-wide informed consent and individual informed consent. All eligible patients with malaria consented to participate in the study. While thick smears were Giemsa-stained and immediately read for initial parasitaemia screening and quantification, thin smears were used for parasite speciation to identify *P. malariae* species carriage. For the *ex vivo* drug assay, 3–10 mL of venous blood was taken before any antimalarial medication and filter-paper-dry blood spots were made for onward DNA extraction for the PCR assays. Only samples with ring stage >80% were eligible for the drug assay.

Laboratory procedures and data analysis

PCR assay

Genomic DNA was extracted from dried blood spots using the QIAamp DNA Mini Kit only (QIAgen, Valencia, CA, USA), following the manufacturer's instruction. PCR assay was conducted for *Plasmodium* species differentiation using the small subunit ribosomal RNA 18S gene.[16] We used the most advanced and reliable PCR techniques for the diagnosis of human malaria parasite species.[17]

We performed two nested PCR assays. The first for the diagnosis of the genus *Plasmodium* using the following genus-specific set of primers.[17] Set 1 was: rPLU1 (TCAAAGATTAAGCCATGCAAGTGA) and rPLU5 (CCTGTTGTTGCCTTAAACTTC). Set 2 was: rPLU3 (TTTTTATAAGGATAACTAC GGAAAAGCTGT) and rPLU4 (TACCCGTCATAGCCATGTTAGGCCAATACC). The second nested PCR was to determine specific *Plasmodium* species using the following set of primers: Set 1 as above and Set 3: rFAL1 (TTAAACTG GTTTGGGAAAACCAAATATATT) and rFAL2 (ACACAATGAACTCAATCATGAC TACCCGTC) for *P. falciparum* (206 bp); rVIV1 (CGCTTCTAGCTTAATCCACATA ACTGATAC) and rVIV2 (ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA) for *P. vivax* (121 bp); rMAL1 (ATAACATAGTTGTACGTTAAGAATAACCGC) and rMAL2 (AAAATTCCCATGCATAAAAAATTATACAAA) for *P. malariae* (145 bp); rOVA1 (ATCTCTTTTGCTATTTTTTAGTATTGGAGA) and rOVA2 (ATCTAAGAATTTCACC

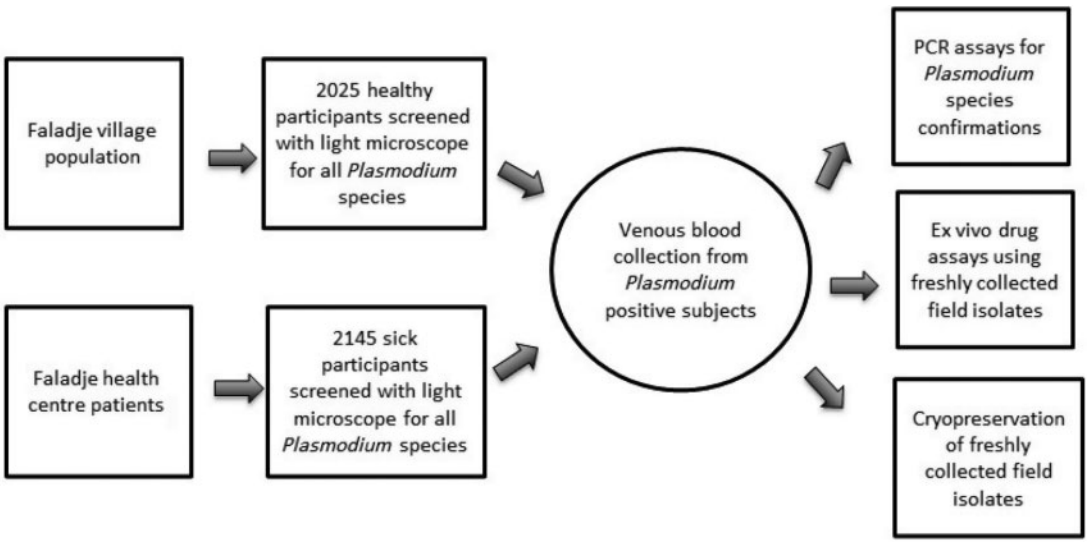

Figure 1. Study design scheme. Faladje village population and health centre patients were screened by light microscopy for malaria parasite species, followed by venous blood draw from malaria parasite carriers. Freshly collected venous blood was used each for *ex vivo* drug assay, cryopreservation and for PCR assays.

TCTGACATCTG) for *P. ovale curtisi* (787–789 bp); and rOVA1v (ATCTCCT TTACTTTTTGTACTGGAGA) and rOVA2v (GGAAAAGGACACTATAATGTATCC TAATA) for *P. ovale wallikeri* (782 bp). Twenty-five μL of reaction mixture contained: 0.025 U Taq DNA polymerase, 2.5 mM magnesium chloride, 200 μM each dNTP and 1 μM each primer. Genus *Plasmodium* diagnosis PCR was run for 25 cycles whereas specific *Plasmodium* species PCR was done using 30 cycles. The PCR amplicons were separated by electrophoresis using a 2% agarose gel. The corresponding typical bands of the different species were used to identify each species. The mono-infection samples were confirmed for *ex vivo* drug assay validation.

Ex vivo drug assay

The compounds tested included chloroquine, lumefantrine, artemether and piperaquine as reference compounds (all from Sigma), the GNF179, a close analogue of KAF156 imidazolopiperazines,[18,19] and the *Plasmodium* phosphatidylinositol-4-OH kinase (PI4K)-specific inhibitor KDU691[20,21] as clinical candidates (both provided by Novartis). For the *ex vivo* drug assay, 3–6 h freshly collected venous blood of *P. malariae* and *P. falciparum* asexual stages were used for parasite cultivation and antimalarial evaluation as previously described.[18,22] *P. falciparum* culture conditions and media without glucose supplement were used for *P. malariae* cultivation. While a 48 h standard SYBR Green assay was used for *P. falciparum*, this assay time was extended to 72 h for *P. malariae* because of the longer life cycle of this parasite. In a prolonged drug assay, *P. falciparum* was incubated for 72 h while *P. malariae* was incubated for 96 h. Optimal standard and prolonged SYBR Green assay using fresh isolates were performed in two separate assay plates for, respectively, 72 and 96 h timepoints systematically for all *P. malariae* isolates. For *P. falciparum* fresh isolates, drug assay was done using 48 and 72 h timepoints systematically. Ten mM DMSO stock compounds were 1/3 serially diluted, starting at 10 μM, into eight concentration points and tested in duplicated wells. At least 10 independent isolates were tested against each compound. Fluorescence data were plotted using GraphPad Prism 8. The data were curve fit with a variable slope function to estimate IC_{50} values. For each isolate, a Z′ factor to assess assay quality was calculated from positive controls (eight drug-free wells) and negative controls (eight parasite-free, red blood cell control wells). Z′ values over 0.5 were considered good assays, but each curve was examined by eye for suitability. Some assays with Z′ below 0.5 may be considered valid depending on factors such as the standard error of the curve fit IC_{50}. Dose–response curves and IC_{50} were calculated by non-linear regression analysis using GraphPad Prism software version 8, with the data previously normalized to the untreated controls. The statistical test (i.e. *t*-test) was done using GraphPad Prism software version 8. A P value <0.05 was considered to be significant.

Ethics

The current study protocol was reviewed and approved by the ethics committees of the Faculties of Medicine-Odonto-Stomatology and Pharmacy, University of Science and Techniques and Technology of Bamako, Mali with the reference numbers 2017/141/CE/FMPOS and 2019/168/CE/FMPOS/FAPH. Only participants or their parent/guardian who provided written informed consent, plus children able to understand the study who gave assent were enrolled in this study. All patients with malaria that consented to participate in the study were enrolled and treated using recommended artemether/lumefantrine therapy or quinine in case of artemether/lumefantrine failure to clear the parasite.

Results

P. malariae causes symptomatic malaria and is highly frequent in non-symptomatic infections

Over 4000 participants distributed into symptomatic ($n = 2145$) and non-symptomatic ($n = 2025$) populations were screened in this study (Figure 1). Participants were enrolled during the low-transmission seasons (January to March and October to December), enabling the detection of mono-infections of non-*falciparum* species (Figure 2a). *P. falciparum* was highly detected mainly in symptomatic participants at the end of the high-transmission malaria season from October to December, similar to *P. ovale* (Figure 2b and d). Unlike *P. falciparum* and *P. ovale*, cases of *P. malariae* were mainly detected from January to March, predominantly in asymptomatic participants (Figure 2c). In symptomatic participants, *P. falciparum* was the most frequent species (96.84%), followed by *P. malariae* (2.38%), *P. ovale* (0.15%) and mixed infections with *P. falciparum* (0.63%) (Table 1). When the prevalence was evaluated in the same symptomatic population, it represented 58.6%, 1.44% and 0.09%, respectively, for *P. falciparum* followed by *P. malariae* and *P. ovale* (Table 1). In non-symptomatic participants, *P. malariae* infections were more frequent (14.53%) than those in symptomatic participants (2.38%) (Table 1). Interestingly, lower *P. falciparum* infections were observed in non-symptomatic participants (83.03%) as compared with that in symptomatic participants (96.84%) (Table 1). The frequency and prevalence of *P. ovale* infections were less than 1% in both symptomatic and non-symptomatic populations (Table 1).

Compromised efficacy of currently used antimalarials against P. malariae

We aimed to assess the susceptibility of freshly collected *P. malariae* clinical field isolates to the available and currently used antimalarials using a 72 h *ex vivo* drug assay according to the 72 h blood stage maturation life cycle of this *Plasmodium* strain [Figure 3a–d (Panel 1) and Table 2]. For comparison purposes, we included freshly collected *P. falciparum* clinical field isolates and the chloroquine-resistant laboratory-adapted strain 7G8. Compounds were tested on *P. falciparum* using the 48 h *ex vivo* drug assay according to its 48 h blood stage life cycle [Figure 3a–d (Panel 1) and Table 2]. Artemether and lumefantrine were highly inhibitory to *P. falciparum* isolates and 7G8 with IC_{50}s in the nanomolar range (Figure 3a and b). Unlike *P. falciparum*, a significant number (approximately half) of *P. malariae* field isolates displayed very high IC_{50}s while the other half was susceptible to artemether and lumefantrine ($P = 0.0034$ and $P = 0.0012$, respectively) (Figure 3a and b, Tables 2 and 3). Chloroquine, as expected, failed to efficiently inhibit the known chloroquine-resistant 7G8 *P. falciparum* laboratory-adapted strain displaying an elevated IC_{50} as compared with artemether and lumefantrine (Figure 3c). When used against clinical isolates of *P. malariae* and *P. falciparum*, only half of the tested isolates were susceptible to chloroquine for each species (Figure 3c). Thus, *P. malariae* showed no difference in drug susceptibility when compared with *P. falciparum* following chloroquine exposure ($P = 0.9367$) (Figure 3c). In conditions when no drug response was detected against a field isolate, 10 μM used as the maximal concentration for the dose-response drug assay was

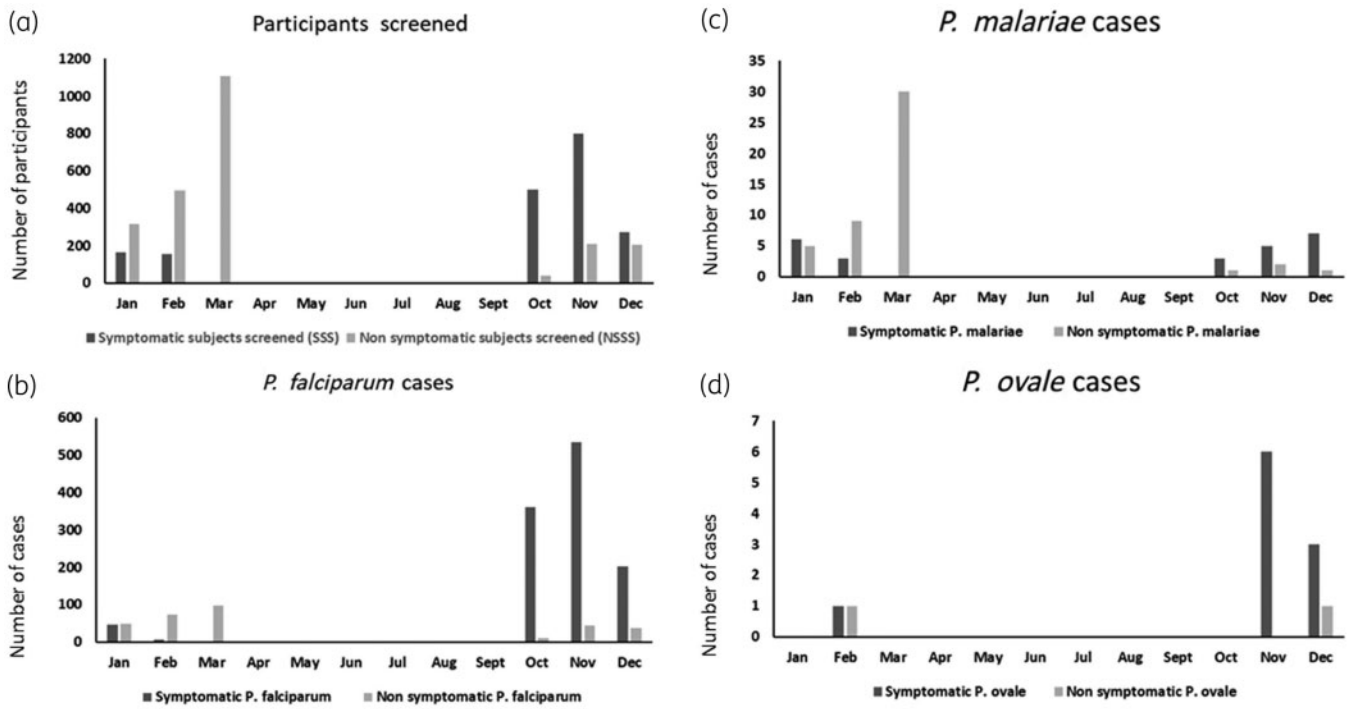

Figure 2. (a) Participants enrolled monthly in the study; (b) monthly *P. falciparum* cases; (c) monthly *P. malariae* cases; and (d) monthly *P. ovale* cases. Dark grey and light grey bars are, respectively, symptomatic and non-symptomatic participants or cases.

Table 1. Summarized PCR-confirmed *P. malariae* prevalence and frequency in symptomatic and non-symptomatic populations

		Pf	Pm	Pow	Pf + Poc + Pm	Pf + Pm	Pf + Pow	Pf + Poc	All species
Symptomatic participants	prevalence	58.6	1.44	0.09	0.04	0.27	0.04	0	60.51
	frequency (%)	96.84, $N=1257$	2.38, $N=31$	0.15, $N=2$	0.07, $N=1$	0.46, $N=6$	0.07, $N=1$	0	100, $N=1298$
Non-symptomatic participants	prevalence	11.85	2.07	0.04	0	0.24	0	0.049	14.27
	frequency (%)	83.04, $N=240$	14.53, $N=42$	0.34, $N=1$	0	1.73, $N=5$	0	0.34, $N=1$	100, $N=289$

Pf, *P. falciparum*; Pm, *P. malariae*; Pow, *P. ovale walikeri*; Poc, P. *ovale curtisi*.

considered by default as the IC_{50} value. The isolates without a drug response (Table 2) were those included in the prolonged drug assay (Table 3). While significant reduced susceptibility was found for *P. malariae* following artemether, lumefantrine and chloroquine exposure, a similar drug response was only observed in *P. falciparum* field isolates that were treated with chloroquine, reflecting the proportion of chloroquine-resistant *P. falciparum* parasites (Figure 3a–c, Tables 2 and 3). Unlike these three compounds, piperaquine efficiently inhibited both *P. malariae* and *P. falciparum* field isolates with no significant difference and potent IC_{50} values that were all under 100 nM ($P=0.7345$) (Figure 3d). To ensure reduced susceptibility of non-susceptible isolates from Figure 3a–c (Panel 1), a prolonged 96 h *P. malariae* and a 72 h *P. falciparum* assay was conducted [Figure 3e–g (Panel 2) and Table 3]. Against *P. malariae*, the three compounds artemether, lumefantrine and chloroquine were more potent in a 96 h prolonged assay (Figure 3e–g, Panel 2). However, the median IC_{50} still remained high with 543.72, 518.88 and 1004.77 nM for artemether, lumefantrine and chloroquine, respectively (Table 3). In this prolonged assay, the median IC_{50} for chloroquine was similar to that found for chloroquine-resistant 7G8 against both *P. malariae* (1004.77 nM) and *P. falciparum* (1265.89 nM) (Table 3).

Advanced discovery compounds, which included the Plasmodium PI4K-specific inhibitor KDU691 and the imidazolopiperazine GNF179, potently inhibited P. malariae clinical isolates

When the laboratory strain 7G8 *P. falciparum* was exposed to KDU691, the drug was highly inhibitory (IC_{50} median = 24.91 nM). Against field isolates, KDU691 efficiently inhibited both *P. malariae* (IC_{50} median = 66.56 nM) and *P. falciparum* (IC_{50} median = 48.015 nM) (Figure 4a). Interestingly like KDU691, the imidazolopiperazine

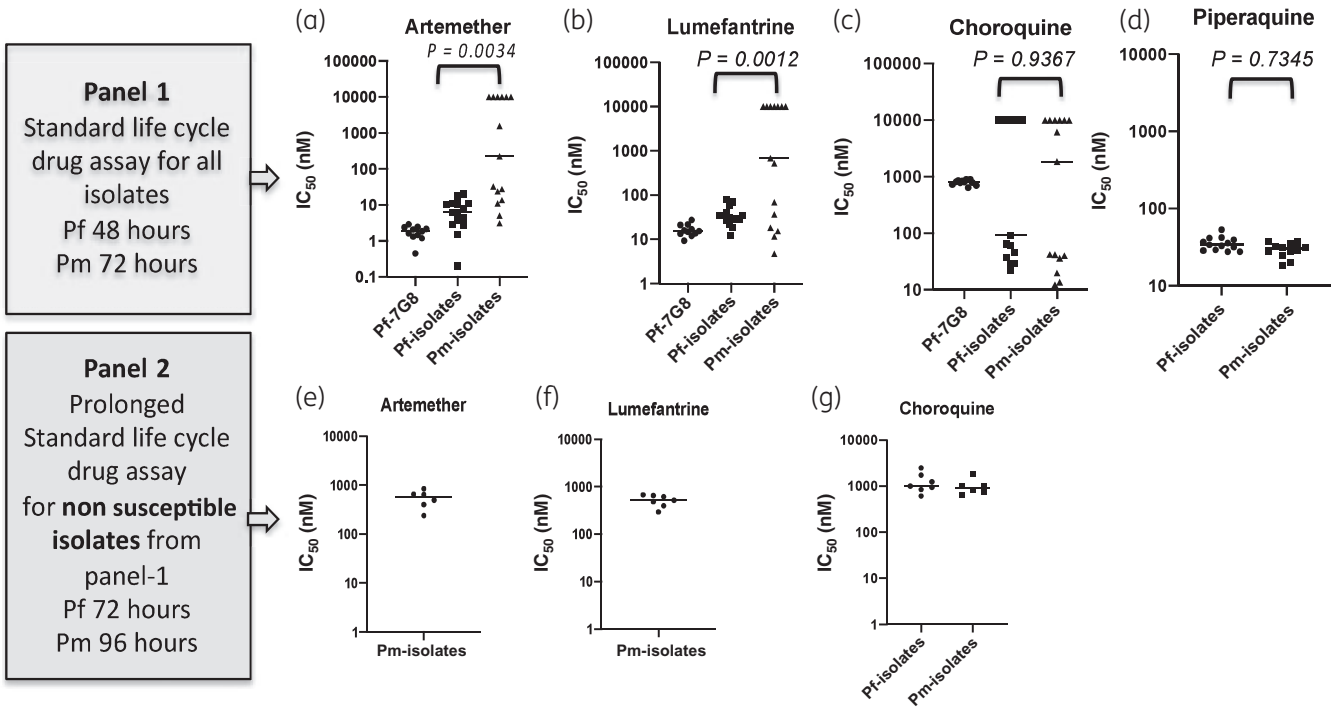

Figure 3. Drug susceptibility of freshly collected field isolates of *P. malariae* compared with that of *P. falciparum* isolates and 7G8 laboratory strain. IC_{50} of (a) artemether, (b) lumefantrine, (c) chloroquine and (d) piperaquine against *P. malariae* and *P. falciparum*. IC_{50} of (e) artemether, (f) lumefantrine and (g) chloroquine from 24 h prolonged drug assay against Panel 1 non-susceptible *P. malariae* and *P. falciparum* field isolates. Median IC_{50} bars are displayed in all graphs for each compound. Pf, *P. falciparum*; Pm, *P. malariae*.

Table 2. Summarized IC_{50} from standard life cycle asexual blood stage drug assay for all isolates and *P. falciparum* 7G8

	Standard life cycle-based drug assay										
	Parasites										
	7G8 48 h assay			Pf isolates 48 h assay				Pm isolates 72 h assay			
Compounds	Art	Lum	CQ	Art	Lum	CQ	PQ	Art	Lum	CQ	PQ
Isolates tested	11	11	11	15	15	15	13	15	15	15	12
Median IC_{50} (nM)	1.88	15.47	810.02	6.21	31.94	92.04	33.85	227.5	681.3	1836	30.23
Mean IC_{50} (nM)	1.82	16.55	793.91	7.83	36.8	4691.93	35.39	4126.48	4757.19	4545.54	29.34
Range IC_{50} (nM)	0.4–2.9	9.3–27.43	639.2–894.4	0.2–17.9	11.92–82.04	22.18–10 000	27.62–53.37	3.16–10000	4.75–10000	12.26–10000	18.48–37.20

Pf, *P. falciparum*; Pm, *P. malariae*; Art, artemether; Lum, lumefantrine; CQ, chloroquine; PQ, piperaquine.

GNF179 was strongly potent against both *P. malariae* (IC_{50} median = 10.04 nM) and *P. falciparum* (IC_{50} median = 6.37 nM) field isolates as compared with the laboratory strain 7G8 *P. falciparum* (IC_{50} median = 7.38 nM) (Figure 4b). Thus, *P. malariae* susceptibility was not different to that of *P. falciparum* when exposed to KDU691 and the GNF179 treatment ($P = 0.7255$ and $P = 0.2726$, respectively) (Figure 4a and b).

Discussion

In sub-Saharan Africa, malaria has been historically and mainly attributed to *P. falciparum* infections. Current surveillance and diagnostic tools in most sub-Saharan African countries are not designed to identify and report non-*falciparum* infections, such as *P. malariae*, accurately.[23] Thus, this resulted in a dearth of accurate data of *P. malariae* epidemiological and drug susceptibility. Our current study focused on the accurate detection of non-*falciparum* species, such as *P. malariae*, using combined diagnostic tools of microscopy and PCR. We selected lower-transmission seasons when

Table 3. Summarized IC_{50} from 24 h prolonged drug assay for non-susceptible isolates from Panel 1 or Table 2

	Prolonged drug assay for non-susceptible isolates from Panel 1							
	Parasites							
	Pf isolates 72 h assay				Pm isolates 96 h assay			
Compounds	Art	Lum	CQ	PQ	Art	Lum	CQ	PQ
Isolates tested	–	–	7	–	6	7	6	–
Median IC_{50} (nM)	–	–	1006.19	–	566.67	518.61	912.77	–
Mean IC_{50} (nM)	–	–	1265.89	–	543.72	518.88	1004.77	–
Range IC_{50} (nM)	–	–	611.57–1735.3	–	239.88–841.46	296.41–672.04	639.92–1831	–

Pf, *P. falciparum*; Pm, *P. malariae*; Art, artemether; Lum, lumefantrine; CQ, chloroquine; PQ, piperaquine.
A dash represents susceptible field isolates from Panel 1.

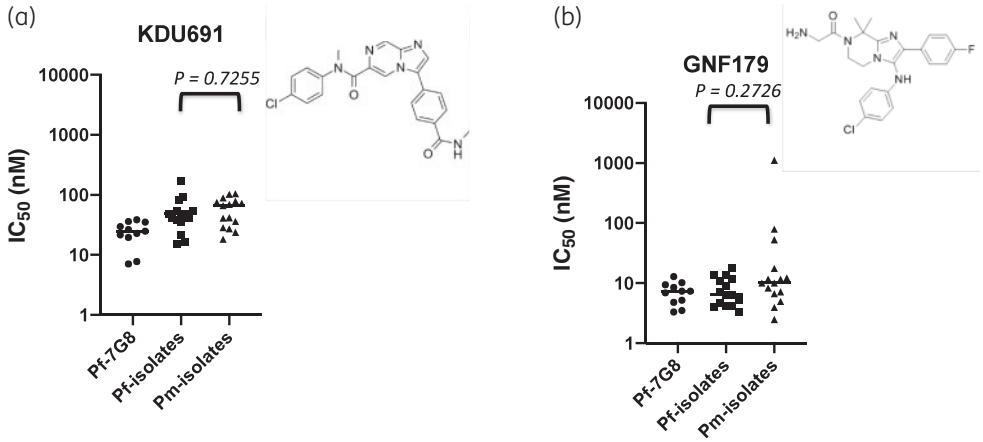

Figure 4. Drug susceptibility of freshly collected field isolates of *P. malariae* compared with that of *P. falciparum* isolates and 7G8 strain. IC_{50} of (a) KDU691 and (b) GNF179 against 15 field isolates of *P. malariae* and 15 field isolates of *P. falciparum*. IC_{50} values are from 11 independent experiments for 7G8 *P. falciparum*. Median IC_{50} bars are displayed in each graph for each compound. Pf, *P. falciparum*; Pm, *P. malariae*.

malaria cases can still be detected (January to March and October to December) to enrol the participants in this study and also to minimize the detection of mixed infections with *falciparum* species (Figure 2a). In April and May, malaria cases are difficult to detect whereas from June to September correspond to high-transmission season of *falciparum* malaria.

While *P. falciparum* was thought to mainly represent all malaria cases, epidemiological data about *P. malariae* significance are lacking, as previously reported for *P. vivax* in sub-Saharan Africa.[24] Our data revealed a high frequency of *P. malariae* infections in the non-symptomatic population while only a few yet important infections causing symptomatic malaria. Like *P. falciparum*, *P. malariae* infections were associated with clinical symptomatic malaria. The high *P. malariae* infection frequency (15%) in the asymptomatic population can result in long-lasting chronic infection if not treated. Such non-treated infections of *P. malariae* may also evolve into hyper-reactive malarial splenomegaly and possibly to a life-threatening complication such as severe illness and may in turn cause death.[25] In addition to the negative clinical impact, *P. malariae* infections may result in an important negative socio-economic impact. Thus, *P. malariae* is highly frequent in sub-Saharan Africa[2–4] and hence requires treatment and adequate elimination tools. Therefore, this study aimed also to identify the possible treatments for *P. malariae* malaria.

To identify such treatments and drug-intervention tools, we tested a panel of reference antimalarials against freshly collected *P. malariae* field isolates as compared with that of *P. falciparum*. With the exception of chloroquine that has been withdrawn in sub-Saharan Africa because of *P. falciparum* resistance,[26,27] all compounds tested were potent against *P. falciparum* isolates. Unlike *P. falciparum*, half of *P. malariae* isolates were not susceptible *ex vivo* to artemether, lumefantrine and chloroquine. All *P. falciparum* isolates tested were susceptible to both artemether and lumefantrine with IC_{50} values comparable to the ones obtained with the 7G8 strain.

This reduced susceptibility of *P. malariae* to artemether and lumefantrine raises the additional hypothesis of *P. malariae* potential resistance or tolerance to these compounds. WGS of these drug-unsusceptible versus -susceptible parasites is required to explore this further and identify any existing drug resistance markers in *P. malariae*. Furthermore, such phenotyping and genotyping need to be coupled with knowledge of clinical outcomes to

comprehensively confirm resistance of *P. malariae*. Drug resistance that may impact artemether/lumefantrine efficacy in the future is now spreading in southeast Asia[28,29] and such additional studies will be important to assess if the same risks are evident in sub-Saharan Africa for *P. malariae*. The US CDC has suggested chloroquine for treating *P. malariae* infections. However, chloroquine has been withdrawn in many sub-Saharan African countries. About half of our tested *P. malariae* isolates were not susceptible to chloroquine. On a prolonged assay, the IC_{50} values were comparable to chloroquine-resistant 7G8 parasites, suggesting chloroquine resistance in both species' isolates. Case reports also indicated chloroquine and other antimalarial treatments failure against *P. malariae* in human subjects[30–32] and *in vitro*.[33] Further studies linking chloroquine clinical failure to a genotype encoding mutations known to confer chloroquine resistance (*Pmcrt*), and any other genetic factors responsible for reduced *ex vivo* susceptibility, would be necessary to conclusively prove chloroquine-resistant *P. malariae* isolates are also circulating in this area of Africa.

In a previous report,[34] artemether/lumefantrine treatment failure in a human with *P. malariae* infection was not associated with Kelch13 mutations. One possible explanation for artemether/lumefantrine treatment failures that has been put forward is that the 3 day regimen could be sub-optimal for *P. malariae* because of its long 72 h intra-erythrocytic asexual life cycle.[34,35] *P. malariae* also has a prolonged erythrocytic life cycle. Thus, because ACTs and other treatments for *P. falciparum* have been developed based on a *P. falciparum* 48 h life cycle and *P. falciparum* drug susceptibility, their use in the treatment of *P. malariae* infections may be inadequate.[34] Artemether and lumefantrine are the first-choice treatment for uncomplicated *P. falciparum* malaria and the most widespread malaria treatments in sub-Saharan Africa. These data suggest that based on both *ex vivo* reduced drug susceptibility and a potential sub-optimal drug concentration, which is usually designed based on the life cycle duration of *P. falciparum*, artemether and lumefantrine may not be appropriate for treating *P. malariae* infections.

In contrast, piperaquine highly inhibited both *P. malariae* and *P. falciparum* isolates. Piperaquine was used for uncomplicated malaria as monotherapy to replace chloroquine in China in response to chloroquine resistance in the 1970s. However, piperaquine resistance has rapidly emerged resulting in the cessation of its use in China in the 1980s and suggested cross-resistance with chloroquine.[36] Our *ex vivo* chloroquine-unsusceptible isolates were all efficiently inhibited by piperaquine, which supports a previous report that displayed no association between piperaquine susceptibility and chloroquine resistance.[37] Thus, DHA–piperaquine could be used as a rescue treatment for *P. malariae* infections in areas with no reported piperaquine resistance in *P. falciparum*. Also, the novel class of antimalarials tested in the assay including GNF179 and KDU691 all proved potent against *P. malariae*. These are from novel classes of antimalarials bearing excellent properties and may be representative of the advanced candidates of each class KAF156 and MMV390048 in Phase II clinical trials.[38–42] Candidate drugs with new mode of action such as imidazolopiperazines and the PI4K-specific inhibitor could be developed as efficacious against all *Plasmodium* species. Our data have revealed that *P. falciparum* treatments can be identified and potentially repurposed to effectively inhibit and treat *P. malariae*. The relevance of our current data for future first-line policies against uncomplicated *P. malariae*, *P. falciparum* or their mixed infections malaria would be to guide proper and efficient management and treatment of *P. malariae* mono- and mixed infections with *P. falciparum*. The drug susceptibility data could influence country policies on disease management and prevention and even may go further to influence curricula for health professionals. Also, the identification of clinical candidate antimalarials bearing new mode of action against *P. malariae* can be exploited for better drug candidate selection that can inhibit all *Plasmodium* species and prioritized for clinical development.

Conclusions

In sub-Saharan Africa where malaria is most prevalent and the medical need is the greatest, *P. malariae* is being increasingly detected with reduced susceptibility to current treatments. Through this current study, we detected a high frequency of *P. malariae* infections in non-symptomatic populations and a low frequency in patients bearing clinical symptoms. We also profiled *P. malariae* drug susceptibility and identified piperaquine as a potent inhibitor of all *P. malariae* isolates, suggesting that it could be a component in a possible rescue treatment combination. Our study revealed that certain ACTs such as artemether/lumefantrine efficacy against *P. malariae* could be compromised, with additional studies needed to definitively conclude on this. We also demonstrated that close analogues (imidazolopiperazine GNF179 and PI4K inhibitor KDU691) from two clinically efficacious compounds, KAF156 and MMV390048, are active against *P. malariae* and the deadliest *P. falciparum* and could be considered for development as potential treatment for both *P. malariae* and *P. falciparum* infections. Therefore, these novel antimalarials would be excellent tools to target the elimination of both *P. malariae* and *P. falciparum*.

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Transparency declarations

B.C. is a member of staff of Medicines for Malaria Venture (MMV). All other authors: none to declare. The views expressed in this article do not necessarily represent the decisions, policy, or views of the MMV.

Author contributions

L.D., Y.A., N.D., F.S., C.P.O.S., A.S.H., A.T. and S.A.S.D. co-designed and performed the experiments. L.D. and Y.A. analysed the data. M.D. provided access to some important laboratory facilities. L.D., A.A.D., Y.A. and G.A.A. co-designed the project and wrote the manuscript. All authors critically reviewed the manuscript.

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