Longitudinal ex vivo and molecular trends of chloroquine and piperaquine activity against *Plasmodium falciparum* and *P. vivax* before and after introduction of artemisinin-based combination therapy in Papua, Indonesia

Jutta Marfurt\(^a,^b,^*\), Grennady Wirjanata\(^a,^2\), Pak Prayoga\(^b\), Ferryanto Chalfein\(^b\), Leo Leonardo\(^b\), Boni F. Sebayang\(^c\), Dwi Apriyanti\(^c\), Maic A.E.M. Sihombing\(^c\), Leily Trianty\(^c\), Rossarin Suwanarusk\(^a,^1\), Alan Brockman\(^a\), Kim A. Piera\(^a\), Irene Luo\(^a\), Angela Rumaseb\(^a\), Barbara MacHunter\(^a\), Sarah Auburn\(^a,d,e\), Nicholas M. Anstey\(^b\), Enny Kenangalem\(^b,f\), Rintis Noviyanti\(^c\), Bruce Russell\(^a,^i\), Jeanne R. Poespoprodjo\(^b,g\), Ric N. Price\(^a,d,e\)

\(^a\) Global and Tropical Health Division, Menzies School of Health Research, Charles Darwin University, PO Box 41096, Casuarina, NT, 0811, Darwin, Australia
\(^b\) Pusparum Health and Community Development Foundation (PHCDF), Jl. Caritas No. 1, 99961, Timika, Papua, Indonesia
\(^c\) Eijkman Institute for Molecular Biology, Jl. Diponegoro 69, 10430, Jakarta, Indonesia
\(^d\) Centre for Tropical Medicine and Global Health, Nuffield Department of Medicine, University of Oxford, UK
\(^e\) Mahidol-Oxford Tropical Medicine Research Unit, Mahidol University, Bangkok, Thailand
\(^f\) District Health Authority, Timika, Papua, Indonesia
\(^g\) Paediatric Research Office, Faculty of Medicine, Public Health and Nursing, Gadjah Mada University, Yogyakarta, Indonesia

**ABSTRACT**

Drug resistant *Plasmodium* parasites are a major threat to malaria control and elimination. After reports of high levels of multidrug resistant *P. falciparum* and *P. vivax* in Indonesia, in 2005, the national first-line treatment policy for uncomplicated malaria was changed in March 2006, to dihydroartemisinin-piperaquine against all species.

This study assessed the temporal trends in ex vivo drug susceptibility to chloroquine (CQ) and piperaquine (PIP) for both *P. falciparum* and *P. vivax* clinical isolates collected between 2004 and 2018, by using schizont maturation assays, and genotyped a subset of isolates for known and putative molecular markers of CQ and PIP resistance by using Sanger and next generation whole genome sequencing.

The median CQ IC\(_{50}\) values varied significantly between years in both *Plasmodium* species, but there was no significant trend over time. In contrast, there was a significant trend for increasing PIP IC\(_{50}\) in both *Plasmodium* species from 2010 onwards. Whereas the South American CQ resistant 7G8 pfcrt SVMNT isoform has been fixed since 2005 in the study area, the pfmdr1 Y86Y allele frequencies decreased and became fixed at the wild-type allele in 2015. In *P. vivax* isolates, putative markers of CQ resistance (no pvcrt-o AAG (K10) insertion and pvmdr1 Y967F and F1076L) were fixed at the mutant alleles since 2005. None of the putative PIP resistance markers were detected in *P. falciparum*.

The ex vivo drug susceptibility and molecular analysis of CQ and PIP efficacy for *P. falciparum* and *P. vivax* after 12 years of intense drug pressure with DHP suggests that whilst the degree of CQ resistance appears to have been sustained, there has been a slight decline in PIP susceptibility, although this does not appear to have reached clinically significant levels. The observed decreasing trend in ex vivo PIP susceptibility highlights the importance of ongoing surveillance.
1. Introduction

Malaria remains one of the most important tropical diseases globally, causing more than 200 million clinical cases per year and more than 400 deaths (WHO, 2019). The emergence and spread of resistant Plasmodium parasites are a major threat to disease control and elimination efforts. The Greater Mekong Subregion (GMS) has been the epicentre of the emergence of P. falciparum resistance with parasites now being resistant to almost all antimalarials used to date (Dondorp et al., 2009; Noedl et al., 2009; Wongsriratchalai et al., 2002), including the artemisinin-based combination therapy (ACT) regimen dihydroartemisinin-piperaquine (DHP) (Saunders et al., 2014). Standardized clinical and in vitro protocols have been developed for P. falciparum, which have enhanced the surveillance of resistant parasites and facilitated discovery and validation of the underlying genetic mechanisms of resistance and complementary molecular surveillance. In contrast, the surveillance of drug resistant P. vivax is constrained to clinical efficacy studies that are confounded by various host factors such as host immunity and drug metabolism, as well as difficulties in pinning down the origin of recurrent parasites as recrudescent (drug resistant), relapsing (originating from hypnozoites), or new infections (Popovic and Menard, 2015).

P. falciparum can be maintained in continuous in vitro culture, making it amenable to drug susceptibility testing by exposure to the presence of serial drug concentrations. In contrast, P. vivax preferentially invades young red blood cells and its limited reproductive capacity has so far prevented continuous in vitro culture. Although drug susceptibility of P. vivax has been assessed using short-term ex vivo culture, such studies generally require blood collected directly from patients with malaria and thus, is limited to field sites with good laboratory infrastructure. The life cycle stage composition of P. vivax infections is highly variable and can potentially bias ex vivo drug susceptibility testing results (Russell et al., 2008; Thomson-Luque et al., 2019). The inability to propagate P. vivax parasites in continuous in vitro culture has also severely hampered investigation of the molecular determinants of drug resistance of the parasite. To date, putative determinants of drug action and resistance have been identified by assessing ex vivo drug susceptibility profiles (Russell et al., 2008), adaptation and propagation of P. vivax isolates in non-human primate hosts (McCallum et al., 2020), expression of orthologous genes identified in P. falciparum in heterologous systems (Sa et al., 2006), and correlation of putative molecular markers and in vivo or ex vivo CQ-resistant phenotypes (Marfurt et al., 2008; Suwanarusk et al., 2007). However, results of validation studies have been inconclusive and hence, a consensus set of resistance markers does not exist.

In Papua Indonesia, high levels of antimalarial drug resistance have emerged in both P. falciparum and P. vivax. Clinical trials conducted in 2004 demonstrated that more than 48% of patients with P. falciparum malaria treated with sulfadoxine-pyrimethamine plus chloroquine and 65% of patients with P. vivax malaria treated with chloroquine alone had recent infections within 28 days (Ratchiff et al., 2007). In March 2006, antimalarial treatment guidelines were revised to a universal policy of dihydroartemisinin-piperaquine (DHP) for uncomplicated malaria due to any species.

This study aimed to assess the temporal trends in ex vivo drug susceptibility to chloroquine (CQ) and piperaquine (PIP) for both P. falciparum and P. vivax clinical isolates collected between 2004 and 2018 and associated patterns of known and putative molecular markers of antimalarial drug resistance.

2. Materials and methods

2.1. Study area and field sample collection

The study was conducted in Timika, southern Papua Province in Eastern Indonesia, between March 2004 and October 2018. Patients presenting to the Rumah Sakit Mitra Masyarakat (RSMM) and Rumah Sakit Umum Daerah (RSUD) hospitals were screened for eligibility criteria and were asked to enrol into the study if they had a microscopically confirmed P. falciparum or P. vivax mono-species infection and a parasitaemia between 2000 and 80,000 parasites/μL. Between March 2004 to December 2008, Plasmodium spp. isolates were processed from all eligible patients, but from December 2008 isolates were processed only if the blood film at screening had at least 66% of asexual forms at ring stage. Patients were excluded from the study if they were treated with CQ in the last 30 days or any other antimalarials in the previous two weeks or had haemoglobin levels below 5 g/dL. After written informed consent was obtained, 5 mL of venous blood was collected and processed immediately. Host white blood cell depletion was performed by using either CF11 cellulose filtration (Richards and Williams, 1973); March 2004–April 2015) or commercially available Plasmodipur® filters (Europroxima B.V., Arnhem, The Netherlands; May 2015–October 2018) according to the manufacturer’s instructions. Packed infected red blood cells (iRBCs) were used for all ex vivo drug susceptibility assays.

2.2. Compounds and drug plate preparation

A range of novel antimalarial agents were assessed and have been reported previously (Baragana et al., 2015; Brunschwig et al., 2018; Kuhnen et al., 2014; Le Bihan et al., 2016; Marfurt et al., 2011a; Marfurt et al., 2011b; Marfurt et al., 2012b; Nilson et al., 2013; O’Neill et al., 2017; Phillips et al., 2016; Price et al., 2010; Vaidya et al., 2014; Wirjanata et al., 2015a; Wirjanata et al., 2015b; Xue et al., 2019). All plates also contained the two standard antimalarials chloroquine (CQ) and piperaquine (PIP). CQ and PIP were sourced from the WWARN QA/QC Reference Material Programme (Lourens et al., 2010). Drugs were prepared as 1 mg/mL solution in dimethyl sulfoxide (DMSO) and drug plates pre-dosed by diluting the compounds in 50% methanol followed by lyophilisation and storage at 4 °C. Each 96- well drug plate contained 11 serial 2-fold dilutions in duplicate with a maximum concentration of 2993 nM for CQ and 1146 nM for PIP.

2.3. Ex vivo drug susceptibility assay

CQ and PIP susceptibilities of Plasmodium laboratory strains and clinical isolates were measured using a modified schizont maturation assay as described previously (Marfurt et al., 2012a; Wirjanata et al., 2017). Drug susceptibility profiles were presented as inhibition of parasite growth from ring to schizont stage. Two hundred microliters of a 2% hematocrit blood medium mixture (BMM), consisting of RPMI 1640 medium supplemented with 10% human serum (P. falciparum) or McCoy’s 5A medium (Life Technologies, VIC, Australia) supplemented with 20% human serum (P. vivax), were added to each well of the pre-dosed 96-well drug plates. The parasites were cultured in a candle jar at 37.0 °C for 35–50 h and growth in the control well monitored. Incubation was deemed successful and stopped when ≥40% of ring-stage parasites had reached the mature schizont stage (≥4 distinct nuclei per parasite) in the drug-free control well. The assays were harvested by preparing thick blood films from each well of the plates; slides were stained with 5% Giemsa solution for 30 min and examined microscopically. The number of schizonts per 200 asexual stage parasites was determined for each drug concentration and normalized to the control well (Marfurt et al., 2011a, 2011b).

2.4. Quality control (QC) procedures

Drug plate quality was assessed using duplicate independent schizont maturation assays with the chloroquine-resistant P. falciparum laboratory strain K1 and the chloroquine-sensitive strain PC27. The P. falciparum strains (BEI Resources, ATCC Manassas, Virginia, US) were maintained using the method of Trager and Jansen (Trager and Jensen,
IC50 values were estimated from IC50 values and the Hill slope (i.e., gamma) of the sigmoid drug response curve using the formula: IC50 = (IC95) + (IC5). Only data derived from drug plates passing independent quality control were included in the analysis. Previous threshold analysis has demonstrated that reliable drug response data from schizont maturation assays require >66% of parasites to be at ring stage at the start of the assay with a duration of drug exposure exceeding 34 h (Kerlin et al., 2012). IC50 values for CQ were also categorised to those above and below 100 nM for *P. falciparum* (Cerutti et al., 1999) and 220 nM for *P. vivax*, with the latter cut-off value determined at the 35th percentile of the continuous non-linear curve of ranked IC50 values from the region in 2005 when clinical CQ failure rates had reached 65% (Swanaranus et al., 2007).

Statistical analysis was performed using STATA (version 15.1; Stata Corp., College Station, TX, US) and GraphPad Prism (version 8; GraphPad Software, Inc., San Diego, CA, US). The Mann-Whitney U test, Wilcoxon Signed Rank Test, Spearman Rank correlation, and Mann-Kendall test were used for nonparametric comparisons and correlations.

### 2.7. Ethical approval

Ethical approval for the study was obtained from the Eijkman Institute Research Ethics Commission, Eijkman Institute for Molecular Biology, Jakarta, Indonesia (EIREC-47, EIREC-67, and EIREC-75), the Ethics committee of the National Institute of Health Research and Development, Indonesian Ministry of Health, Jakarta, Indonesia (NIHRD: KS.01.01.6.591, NIHRD: KS.02.01.2.4579, NIHRD: KS.02.01.2.1.4042, NIHRD: KS.02.01.2.1.1615 and NIHRD:23 LB.03.02/KE/4099/2007), and the Human Research Ethics Committee of the Northern Territory (NT) Department of Health & Families and Menzies School of Health Research, Darwin, Australia (MSHR: 02/55, MSHR: 07/06, MSHR: 03/64, MSHR: 05/16, MSHR: 07/14 and HREC, 2010-1396).

### 3. Results

#### 3.1. Ex vivo susceptibility to CQ and PIP

Between 2004 and 2018, 1478 patients were enrolled into the study and drug susceptibility testing attempted for 796 *P. falciparum* and 682 *P. vivax* isolates. A total of 1174 (79.4%) assays were successfully harvested with a success rate of 81.9% (652) for *P. falciparum* and 76.5% (522) for *P. vivax* isolates (Fig. 1).

The geometric mean parasitaemia in the successfully harvested isolates ranged from 11,662 to 55,405 asexual parasites µl⁻¹ in *P. falciparum* isolates and 8516 to 33,514 parasites µl⁻¹ in *P. vivax* isolates, with no significant variation over the study period. At the start of the assay, *P. falciparum* isolates were composed of 100% ring stages in almost all cases, whereas for *P. vivax* the median percentage of ring stages varied from 45% in 2004 to ≥90% from 2007 (Supplementary Fig. 1). The median duration of the assay for both species ranged from 20 to 30 h in the first three years 2004–2006 but exceeded 40 h from 2007 onwards (Supplementary Fig. 2).

Overall, acceptable *P. falciparum* IC50 estimates were available in 97.9% (462/472) isolates for CQ and 97.7% (461/472) for PIP. The corresponding values for *P. vivax* were 97.7% (344/352) for CQ and 96.9% (341/352) for PIP, respectively (Fig. 1). The IC50 values for CQ and PIP are presented in Table 1 and Figs. 2 and 3; the corresponding IC50 values in Table 2 and Supplementary Figs. 3 and 4.

For the 344 *P. vivax* isolates with acceptable CQ data, the median IC50 was 55.4 nM (range: 4.6 to 1411.6; Table 1, Supplementary Fig. 5) and the median IC50 was 125.5 nM (range: 7.6 to 12,127.9; Table 2, Supplementary Fig. 6). A total of 28.8% (99/344) of isolates had an IC50 greater than 100 nM and 5.8% (20/344) an IC50 greater than 220 nM (Swanaranus et al., 2007). The corresponding values for the 462 *P. falciparum* isolates were a median IC50 of 70.2 nM (range: 5.2 to
444.9) and IC_{50} of 139.7 nM (range: 7.3 to 747.2), with 31.0% (143/462) having a CQ IC_{50} greater than 100 nM. The median CQ IC_{50} values varied significantly between years ranging from 36.3 nM in 2008 to 102.9 nM in 2014 for _P. falciparum_, and from 25.5 nM in 2005 to 126.3 nM in 2013 for _P. vivax_, but there was no significant trend over time (Fig. 2).

Of the 341 _P. vivax_ isolates with acceptable PIP data the median IC_{50} was 20.4 nM (range: 1.3 to 220.9) and the median IC_{90} was 38.6 nM (range: 3.0 to 449.9). The corresponding values for the 461 _P. falciparum_ isolates were 23.1 nM (range: 0.3 to 141.4) and 44.9 nM (range: 1.3 to 456.6). There was a significant trend for increasing PIP IC_{50} in both _P. falciparum_ and _P. vivax_ isolates from 2010 onwards (Mann-Kendall (MK) tau: 0.562, _p_ = 0.004; and MK tau: 0.524, _p_ = 0.006, respectively). There was a similar trend for PIP IC_{50} (MK tau: 0.524, _p_ = 0.008; and MK tau: 0.600, _p_ = 0.002, respectively).

In the sensitivity analysis, excluding data from drug plates with outliers in quality control testing and only including isolates with >90% ring stage parasites at the start of the assay and an assay duration of >34 h, the temporal trends for PIP and CQ were similar. There was an increase in PIP IC_{50} for _P. falciparum_ (MK tau 0.543, _p_ = 0.006) and _P. vivax_ (MK tau 0.517, _p_ = 0.012), but not for CQ IC_{50} (MK tau 0.352, _p_ = 0.075 and MK tau 0.257, _p_ = 0.198, respectively; Supplementary Table 1).

### 3.2. Molecular markers of resistance

_Cpf1_ genotypes were available for 27% (131/478) of _P. falciparum_ isolates. In the two years prior to the introduction of DHP (2004 and 2005), the CQ resistant genotype _cpf1_ 72S-V73-M74-N75-76T-220S-326D-333A/S-S-356L/T was present in all isolates and this remained stable over the study time period, except for 2 (1.5%) wild-type isolates, one enrolled in 2011 and the other in 2016 (Table 3). Genotyping data for _pfmdr1_ were available in 44% (203/478) of isolates. The mutant allele 86Y was present in 34% (12/35) of isolates in 2004 and 5% (2/37) in 2014 and thereafter, all parasites were wild-type N86. The allele frequencies of the two other mutant alleles 184F and 1042D increased from 60% (21/35) and 66% (23/35), respectively, prior to the introduction of DHP, to 94% (15/16) in 2017 (Table 4). None of the _P. falciparum_ isolates genotyped for the putative PIP resistance markers had _pfpm2_ copy number amplification or any of the _pfcr_ alleles: 93S, 97Y, 101F, 145I, 218F, 343L, 350B, and 353V, or exo-415G.

Since the _pfcr_ 72S-V73-M74-N75-76T-220S-326D-333A/S-S-356L/T/
**T** was the prevailing genotype and putative PIP resistance markers were not present over the entire study period, genotype-phenotype association analyses were not possible.

Genotyping *pfmdr1* was available in 39% (187/478) of *P. falciparum* isolates and seven genotypes were observed: NFSDD, NYSDS, NYSND, YFSDD, YFSND, YYSDD, and YYSND. The median CQ IC$_{50}$s did not differ between any of these seven genotypes.

A total of 82% (286/350) and 378 *P. vivax* isolates (including 28 isolates without a valid ex vivo CQ susceptibility phenotype) were genotyped for the *pvcrt-o* AAG (K10) insertion and the *pvmdr1* 967 and 1076 alleles, respectively. The *pvcrt-o* K10 insertion in exon 1 was present in 2% (1/46) of samples in 2005–2006, but in none of the 240 samples after 2011. Conversely, the mutant *pvmdr1* alleles 967F and 1076L were present in 96% (123/128) isolates collected between 2005 and 2006, and in all 240 isolates tested after 2011. Since these genotypes were fixed in the *P. vivax* population in the area, association analyses were not possible.

**4. Discussion**

This study presents a comprehensive longitudinal analysis of ex vivo CQ and PIP susceptibility in clinical *P. falciparum* and *P. vivax* field isolates in Papua, Indonesia, where multidrug resistant *P. falciparum* and *P. vivax* co-exist. The analysis comprises more than 1000 clinical isolates.
before and after a change in antimalarial treatment policy in 2006 from CQ for *P. vivax* and CQ + SP for *P. falciparum* to DHP for all patients with uncomplicated malaria due to any species. The results highlight different longitudinal trends. Although there was no change in CQ susceptibility, there was a trend for decreasing PIP susceptibility after 12 years of intense drug pressure with DHP. The lack of a trend for increasing *ex vivo* CQ susceptibility after antimalarial policy change in 2006, contrasts observations in other areas where CQ susceptibility has been monitored before and after changes in national antimalarial policy (Frosch et al., 2014; Hayward et al., 2005). The end of CQ use was not implemented as radically in Papua, Indonesia, as it was in Malawi, Vietnam, and China where the drug was removed from both the public and private sectors. In Papua, more than 40% of patients with malaria continue to seek healthcare in the private sector where CQ remains readily available (Kenangalem et al., 2019). Hence, CQ pressure has likely continued throughout the study period and this may have maintained the continuation of reduced *ex vivo* CQ susceptibility. The increasing PIP IC\(_{50}\)s and IC\(_{90}\)s in Papua for both *Plasmodium* species raises important questions. *Ex vivo* and *in vitro* data from the GMS, where DHP clinical failures have been reported, have shown a two-fold increase in *P. falciparum* PIP IC\(_{50}\) (Agrawal et al., 2017; Chaorattanakawee et al., 2015; Dhingra et al., 2017; Hao et al., 2013; Mwai et al., 2009). Phenotypic characterization of Cambodian parasites

---

**Fig. 3.** Piperaquine susceptibility (IC\(_{50}\)) for *P. falciparum* (open circles, top panel) and *P. vivax* (triangles, bottom panel). Footnote: Data derived from *P. falciparum* and *P. vivax* isolates fulfilling acceptability criteria (>66% rings at the start of the assay and assay duration ≥34 h). The horizontal lines represent median value with vertical lines stretching between the 25th and 75th percentiles.
bias confounding the representativeness of the surveillance data. In our
often in remote malaria endemic settings with limited laboratory
carried out using fresh blood collected directly from infected patients
Year n Amino acid/Allele frequency

| Year | n | P. falciparum | n | P. vivax | n | P. vivax |
|------|---|---------------|---|----------|---|----------|
| 2004 | 8 | 139.7 (77.9–491.3) | 2 | 63.2 (57.6–68.9) | 8 | 29.1 (11.1–57.8) |
| 2005 | 16 | 98.8 (34.0–209.9) | 7 | 524.3 (32.8–441.4) | 14 | 12.8 (9.7–34.3) |
| 2006 | 27 | 70.9 (11.1–147.2) | 12 | 167.9 (7.6–1217.9) | 24 | 23.8 (8.7–71.9) |
| 2007 | 35 | 81.4 (22.3–189.1) | 27 | 114.8 (19.5–5457.7) | 34 | 22.0 (5.3–58.3) |
| 2008 | 41 | 71.6 (7.9–391.8) | 35 | 117.1 (18.6–111.0) | 42 | 22.6 (3.3–146.0) |
| 2009 | 39 | 167.5 (60.4–487.6) | 45 | 151.6 (23.3–318.5) | 39 | 41.8 (6.2–242.4) |
| 2010 | 24 | 168.5 (7.3–418.8) | 13 | 47.4 (30.5–228.4) | 24 | 21.5 (8.0–98.7) |
| 2011 | 44 | 119.3 (13.6–250.2) | 45 | 85.7 (16.9–2831.5) | 44 | 57.1 (20.2–456.6) |
| 2012 | 55 | 177.5 (47.5–648.0) | 36 | 196.2 (42.5–986.4) | 55 | 47.5 (16.3–206.3) |
| 2013 | 21 | 179.7 (55.7–419.5) | 37 | 272.3 (28.2–527.7) | 21 | 58.1 (15.6–110.4) |
| 2014 | 43 | 198.4 (29.8–449.3) | 13 | 232.2 (50.1–4771.8) | 48 | 82.7 (8.9–181.0) |
| 2015 | 32 | 160.4 (17.3–554.3) | 22 | 105.8 (29.5–615.4) | 32 | 70.3 (28.5–311.2) |
| 2016 | 34 | 175.0 (51.8–747.2) | 13 | 106.8 (21.8–270.8) | 34 | 151.8 (20.2–382.2) |
| 2017 | 34 | 178.3 (19.5–550.4) | 30 | 109.2 (26.1–433.0) | 34 | 55.7 (16.0–130.5) |
| 2018 | 9 | 91.6 (14.3–188.7) | 7 | 95.9 (29.8–152.6) | 9 | 43.3 (20.9–67.2) |
Total | 462 | 139.7 (7.3–747.2) | 344 | 125.5 (7.6–1217.9) | 461 | 44.9 (1.3–456.6) |

Footnote: Data derived from *P. falciparum* and *P. vivax* isolates fulfiling acceptability criteria (>66% rings at the start of the assay and assay duration >34 h).

Table 3
Allele frequencies of single nucleotide polymorphisms (SNPs) in *pfcr* over time.

| pfcr | C72S | V73I | M741 | N75D/K | K76T | Y97L | T152A | S163R | A220S | Q271E | N3260/S | T333A/S | I356 L/T | R3711 |
|------|------|------|------|--------|------|------|-------|-------|-------|-------|---------|---------|---------|--------|
| Year | n   | Amino acid/Allele frequency |
| 2004 | 2   | S (1.0) V (1.0) M (1.0) |
| 2005 | 13  | S (1.0) V (1.0) M (1.0) |
| 2006 | 2   | S (1.0) V (1.0) M (1.0) |
| 2011 | 16  | C (0.06) S (0.94) ^a |
| 2012 | 9   | S (1.0) V (1.0) M (1.0) |
| 2013 | 13  | S (1.0) V (1.0) M (1.0) |
| 2014 | 37  | S (1.0) V (1.0) M (1.0) |
| 2015 | 10  | S (1.0) V (1.0) M (1.0) |
| 2016 | 13  | C (0.07) S (0.93) ^a |
| 2017 | 16  | S (1.0) V (1.0) M (1.0) |

^a 95% confidence interval: 0.70–1.0.  
^b 95% confidence interval: 0.66–1.0.

reveals a bimodal drug response for PIP resistant strains (Bopp et al., 2018) and thus, IC<sub>50</sub> or a novel ring stage survival assay (RSA) have been proposed as better indicators of PIP resistance (Duru et al., 2015; Ross et al., 2018). PIP resistant isolates derived using gene-editing or in vitro selected laboratory strains under PIP pressure had IC<sub>50</sub> > 200 nM and IC<sub>50</sub> > 1000 nM (Parobek et al., 2017; Ross et al., 2018). In contrast, in our study the least susceptible Papuan isolate had a PIP IC<sub>50</sub> of 221 nM (IC<sub>50</sub> = 450 nM), with no evidence of a bimodal distribution in drug susceptibility.

Monitoring temporal drug susceptibility trends in clinical Plasmodium isolates is challenging particularly for *P. vivax* which cannot be sustained in in vitro culture. *Ex vivo* P. vivax drug susceptibility has to be conducted using fresh blood collected directly from infected patients often in remote malaria endemic settings with limited laboratory infrastructure. Furthermore, there is potential for significant sampling bias confounding the representativeness of the surveillance data. In our study, the isolates selected for *ex vivo* susceptibility analysis were restricted to those from patients presenting with parasitaemia between 2000 and 80,000 asexual parasites/μL and, for *P. vivax*, greater than 66% of parasites at ring stage at the start of the assay (Kerlin et al., 2012; Russell et al., 2006). Whilst application of these criteria limits the number of eligible isolates, they have been shown to improve the quality of the data, facilitating drug susceptibility testing of novel antimalarial compounds (Baragwala et al., 2015; Le Bihan et al., 2016; Marfurt et al., 2011b; Marfurt et al., 2012b; Nilsen et al., 2013; O’Neill et al., 2017; Phillips et al., 2016; Vaidya et al., 2014; Wirjanata et al., 2015b). The validity of the *P. vivax* *ex vivo* estimates of drug susceptibility is supported by their correlation with poor efficacy in clinical trials (Llanos-Cuentas et al., 2018; Phillips et al., 2016). However, an important limitation of schizont maturation testing is that assays for *P. vivax* isolates are constrained by inability to maintain cultures through schizont rupture and reinvasion. To explore the robustness of our findings a sensitivity analysis was conducted using tighter inclusion criteria (isolates with >90% rings and 34–42 h incubation as well as only isolates
tested on drug plates with narrow quality control IC\textsubscript{50S}). Reassuringly, the temporal trends for PIP remained the same, with no apparent temporal trend in CQ susceptibility.

Previous molecular analyses of clinical isolates of \textit{P. falciparum} collected from a clinical efficacy trial of DHP between 2015 and 2016 showed none of the kelch13 polymorphisms that have been associated with artemisinin resistance (Ariey et al., 2014; Miotto et al., 2015; Poepoprodjo et al., 2018). In conjunction with rapid parasite clearance and cure rates exceeding 90%, this suggests that artemisinin resistance has yet to emerge in the province and the DHA component of the ACT is likely to continue reducing the selective PIP pressure on the parasites. In our current analysis, none of the known molecular markers of PIP resistance were detected in any isolate over the 14-year period.

In the GMS, \textit{pfpm2} copy number variants (CNV) (Amato et al., 2017; Witkowski et al., 2017) and \textit{pfcr} polymorphisms T93S, H97Y, C101F, F145I, I218F M343L, and G353V (Agrawal et al., 2017; Dhingra et al., 2017; Hamilton et al., 2019; Pelleau et al., 2015; Ross et al., 2018; van der Pluijm et al., 2019), and exo-E415G (Amato et al., 2017) occurred almost exclusively on a genetic artemisinin-resistant and the CQ-resistant Southeast-Asian Dd2-type \textit{pfcr} background. Indeed, transfection of \textit{pfpm2} CNVs on the CQ-sensitive 3D7-type \textit{pfcr} background did not result in PIP resistance in vitro (Loesbanluechai et al., 2019). Furthermore, characterization of \textit{pfcr} in field isolates from French Guiana, where CQ was withdrawn in 1995 and the prevalence of CQ resistant \textit{P. falciparum} dropped from >90% to <30% in 2012, revealed persistence of the CQ-resistant South American 7G8-type \textit{pfcr} isoform, but an additional \textit{pfcr} mutation C350R that was associated with decreased PIP susceptibility in vitro (Pelleau et al., 2015).

PIP resistance-associated \textit{pfcr} SNPs in Dd2 and 7G8 strains are associated with PIP efflux from the digestive food vacuole, but reduced CQ transport (Riegel and Roepe, 2020). There was no inverse correlation, suggesting that the interactions between \textit{pfcr} and antimalarials are complex and most likely mediated by additional mutations in \textit{pfcr}, or genetic modifications in other genes. In Timika, the South American 7G8 \textit{pfcr} isoform has been fixed since 2005. As PIP resistance mutations come with a fitness cost and at the expense of losing the CQ-resistant phenotype (Dhingra et al., 2017; Riegel and Roepe, 2020; Ross et al., 2018), it appears that PIP pressure on the parasites remains low, presumably due to the high efficacy of DHA in reducing the exposed parasite biomass.

The temporal patterns of \textit{pfmdr1} SNPs in southern Papua show a different pattern than that of \textit{pfcr}. \textit{Pfmdr1} 86Y, a modulator of CQ resistance (Foote et al., 1990), had a relatively low allele frequency (0.34) in 2005, despite very low CQ efficacy, whereas the frequencies of the 184F and 1042D polymorphisms were higher (0.60 and 0.66, respectively). Over the study period, 86Y allele frequencies declined and became fixed at the wild-type allele in 2015, whereas the frequencies of 184F and 1042D increased and became almost fixed at the mutant alleles in 2017.

The role of PIP selective pressure on \textit{pfmdr1} is unclear. Molecular, clinical and in vitro phenotype association studies and allelic exchange experiments suggest different or no associations between SNPs or \textit{pfmdr1} amplification and PIP resistance (Gil and Krishna, 2017; Veiga et al., 2016). Amato and colleagues investigated the origin and spread of multidrug-resistant \textit{P. falciparum} in South East Asia, and raised the possibility of ‘natural antagonism’ between \textit{pfmdr1} and \textit{pfpm2} amplification (Amato et al., 2017). Interestingly in Papua, Indonesia, no \textit{pfmdr1} CNVs were detected over the entire study period. In-depth analysis of 187 \textit{P. falciparum} isolates for which \textit{pfmdr1} was genotyped (all on the \textit{pfcr} SVMNT background) showed that median CQ IC\textsubscript{50} did not differ between the seven observed genotypes, providing further evidence that \textit{pfmdr1} SNPs are secondary mediators of \textit{P. falciparum} CQ resistance.

Since 2006, there was no temporal trend in the prevalence of putative molecular markers of CQ resistance in \textit{P. vivax}, with polymorphisms in the \textit{pvcrt-o} K10 and \textit{pvmdr1} 967F and 1076I fixed at the mutant alleles, respectively. Hence, the large variation in \textit{ex vivo} CQ susceptibility of \textit{P. vivax} during the study period, in which IC\textsubscript{50} ranged from 4.6 to 855 nM, cannot be explained solely by currently proposed molecular markers of CQ resistance.

In conclusion, the \textit{ex vivo} drug susceptibility and molecular analysis of CQ and PIP efficacy for \textit{P. falciparum} and \textit{P. vivax} after 12 years of intense drug pressure with DHP suggests that whilst the degree of CQ resistance appears to have been sustained, there has been a slight decline in PIP susceptibility, although this does not appear to have reached clinically significant levels. The observed decreasing trend in \textit{ex vivo} PIP susceptibility highlights the importance of vigilance and ongoing monitoring.

### Table 4

Allele frequencies of single nucleotide polymorphisms (SNPs) in \textit{pfmdr1} over time.

| \textit{pfmdr1} | N86Y | Y184F | S1034C | N1042D | D1246Y |
|----------------|------|--------|--------|--------|--------|
| Year           | N    | Amino acid/Allele frequency; 95% Confidence Intervals | N    | Amino acid/Allele frequency; 95% Confidence Intervals | N    | Amino acid/Allele frequency; 95% Confidence Intervals | N    | Amino acid/Allele frequency; 95% Confidence Intervals | N    | Amino acid/Allele frequency; 95% Confidence Intervals |
| 2004           | 35   | N (0.66) | 0.21–0.51 | Y (0.40) | 0.44–0.74 | S (1.0) | N (0.34) | 0.49–0.79 | D (1.0) |
| 2005           | 46   | Y (0.34) | 0.10–0.33 | Y (0.24) | 0.62–0.86 | S (1.0) | N (0.17) | 0.69–0.91 | D (1.0) |
| 2006           | 8    | Y (0.20) | 0.13–0.70 | Y (0.50) | 0.22–0.78 | S (1.0) | N (0.50) | 0.22–0.78 | D (1.0) |
| 2011           | 16   | Y (0.37) | 0.0–0.30 | Y (0.19) | 0.56–0.94 | S (1.0) | N (0.19) | 0.56–0.94 | D (1.0) |
| 2012           | 9    | N (1.0) | F (1.0) | 0.65–1.0 | S (1.0) | D (1.0) |
| 2013           | 13   | N (0.92) | 0.0–0.35 | N (0.08) | 0.65–1.0 | S (1.0) | N (0.08) | 0.65–1.0 | D (1.0) |
| 2014           | 37   | Y (0.08) | 0.01–0.19 | Y (0.08) | 0.78–0.98 | S (1.0) | N (0.08) | 0.78–0.98 | D (1.0) |
| 2015           | 10   | N (1.0) | F (1.0) | 0.57–0.93 | S (1.0) | D (1.0) |
| 2016           | 13   | N (1.0) | N (0.14) | 0.57–0.93 | S (1.0) | N (0.14) | 0.57–0.93 | D (1.0) |
| 2017           | 16   | N (1.0) | Y (0.06) | 0.70–1.0 | S (1.0) | N (0.06) | 0.70–1.0 | D (1.0) |

Declaration of competing interest

None declared.

Acknowledgements

We are grateful to Lembaga Pengembangan Masyarakat Amungme Kamoro, and the Rumah Sakit Mitra Masyarakat (RSM) and Rumah Sakit Umum Daerah (RSUD) Hospitals for their excellent and continuing support in conducting this study.
We thank all the study participants and/or their guardians for consenting to be part of these studies and donating blood for the laboratory experiments.

We are very thankful to our laboratory technicians and assistants who worked hard over the years in the laboratories of the research building of the Papuan Health and Community Development Foundation (PHCDF) and were responsible for every day laboratory maintenance and support, namely Bu Rosmini, Yenny Patontongan, George Warikar, Daud Rumere, Frans Wabiser, Ruland Wansoda, Basbak Mobay, and Chairunisa Fadhilah.

We thank the Australian Red Cross blood transfusion service for the supply of the human sera.

The study was funded by the Wellcome Trust (Senior Research Fellowship in Clinical Science 200909 to RNP), the National Health and Medical Research Council (1023438, 1037304, 1132975 and Fellowship 1135820 to NMA), and the Swiss National Science Foundation (Fellowship for Emerging Researchers: PBSP3-125580, and Advanced Fellowship in Clinical Science 200909 to RNP), the National Health and Community Development Foundation (Papua, Indonesia: a longitudinal surveillance study). PLoS Med. 16, e1002815.

II-III copy number accounts for bimodal piperaquine resistance among Cambodian P. falciparum isolates from the China-Myanmar border to piperaquine and association with artemisinin-resistant Plasmodium falciparum malaria in Cambodia: a genotype-phenotype association study. Lancet Infect. Dis. 17, 164–173.

We refer the reader to the full references section for further details.
candidate with potential for use in prophylaxis, treatment, and prevention of disease transmission. Antimicrob. Agents Chemother. 58, 5060–5067.

Lambros, C., Vanderberg, R.J. 1970. Synthesis of plasmodium falciparum erythrocytic stages in culture. J. Parasitol. 56, 415–420.

Le Bihan, A., de Kanter, R., Arguello-Urbain, T., Binkert, C., Bos, C., Brun, R., Brunner, R., Buchmann, S., Burrows, J., Debruycker, K.J., Delves, M., Ewerling, S., Ferrier, S., Fischl, C., Gano-Benito, L., Giangad, N.F., Heiden, B., Jimenez-Diaz, M.B., Leroy, D., Martinez, M.S., Meyer, S., Moehrle, J.N., Gl. C., Noyvianti, R., Ruecker, A., Sanz, L.M., Sauerwein, R.W., Scheurer, C., Schjoedtkoeck, S., Sinden, R., Snyder, C., Stramer, J., Wirajana, G., Price, R.N., Weller, T., Witters, F., Wdowik, D.A., Chedid, W., Wittlin, S., 2015. Characterization of novel antimalarial compound ACT-415818b: preclinical assessment of activity and dose-fidelity modeling. PLoS Med. 13, e1002138.

Llamasfront, A., Gomis, C., Chiquiut, F., Cen, K., Rens, D., Mijima, T., Toovey, S., Arch, R.H., Phillips, M.A., Rosenberg, F.D., Bath, J., Ng, C.L., Cowell, A.N., Winzeler, E.A., Fidock, D.A., Baker, M., Mohr, J.J., Hooft van Huijsduijnen, R., Gobeau, N., Arnaoutopoulos, N., Andenmann, N., Ruckpul, T., Dupac, S., 2018. Antimalarial activity of single-dose DMS265 in a plasmodium dihydroorotate dehydrogenase inhibitor, in patients with uncomplexed Plasmodium falciparum or Plasmodium vivax malaria infection: a proof-of-concept, open-label, phase 2a study. Lancet Infect. Dis. 18, 874–883.

Locatelli, E., Kotonan, N., de Cozar, C., Chookajorn, T., 2019. Overexpression of plasmepsin II and pfmdr1. Antimicrob. Agents Chemother. 53, 5069–5073.

Fairhurst, R.M., White, N.J., Kwiatkowski, D.P., 2015. Genetic architecture of Plasmodium falciparum and P. vivax. Antimicrob. Agents Chemother. 56, 5073–5078.

Ratcliff, A., Siswantoro, H., Kenangalem, E., Wuwung, M., Brockman, A., Edstein, M.D., 2016. Adaptive evolution of malaria parasites in French Guiana: reversal of chloroquine resistance. J. Infect. Dis. 214, 417–420.

Pelloux, S., Monn, E.L., Dehinga, S.K., Volney, B., Basteras, J., Gubarevich, S.J., Volkman, S.K., Wirth, D.F., Lebrun, E., Fidock, D.A., Neafsey, D.E., Musset, L., 2015. Evolutionary nature of malaria parasites in French Guiana: reversal of chloroquine resistance due to acquisition of a mutation in pfcr. Proc. Natl. Acad. Sci. U. S. A. 112, 11672–11677.

Phillips, M.A., White, K.L., Elbassuoni, S.O., El-Bassuoni, M., Rock, M., Todd, C., van Lingen, F., Heywood, D., Nash, G., Tubman, M.H., Ansbro, M.R., Le Bihan, A., de Kanter, R., Angulo-Barturen, I., Binkert, C., Boss, C., Brun, R., 2012. Comparative ex vivo activity of novel endoperoxides in multidrug-resistant Plasmodium falciparum and P. vivax. Antivir.Agents Chemother. 55, 4461–4464.

McCullagh, F.J., Birrell, G.W., Chavchich, M., Harris, I., Obaldia, N. 3rd, van Breda, K., Marfurt, J., de Monbrison, F., Brega, S., Barbollat, L., Muller, I., Sie, A., Goroti, M., Loesbanluechai, D., Kotanan, N., de Cozar, C., Kochakarn, T., Ansbro, M.R., Le Bihan, A., de Kanter, R., Angulo-Barturen, I., Binkert, C., Boss, C., Brun, R., 2012. Comparative ex vivo activity of novel endoperoxides in multidrug-resistant Plasmodium falciparum and P. vivax. Antivir.Agents Chemother. 55, 4516–4523.

Miotto, O., Amato, R., Ashley, E.A., Macnab, M., Almagro-Garcia, J., Marfurt, J., 2013. Overexpression of plasmepsin II and pfmdr1. Antimicrob. Agents Chemother. 53, 5069–5073.

Smith, D.A., Wirajana, G., Noviyanti, R., Price, R.N., Marfurt, J., Palmer, M.J., Coppo, L.M., Mercer, A.E., Ruecker, A., Delves, M.J., Sinden, R.E., Sieg, P., Davies, F., Rochford, R., Hujug, C.M., Nazon, U.M., Biagini, G., Ward, A., 2017. A tetratose抗formalundaryl drug candidate that overcomes PK13/C580Y dependent antiresistance. Nat. Commun. 8, 15159.

Parek, C.M., Farr, J.B., Brazier, N.F., Lu, C., Chaoattakawatee, S., Gosi, P., Harman, K.S., Norris, L.E., Jofre, J., Proctor, B., Riera, J.E., Saunders, D.L., Lin, J.T., Julião, J.J., 2017. Partner-drug resistance and population substructuring of antiresistant-plasmodium falciparum in Cambodia. Genes (Basel) Bio 9, 566–580.

Pareto, R.D., Amato, R., Aubin, S., Miotto, O., Almagro-Garcia, J., Marfurt, J., Anstey, N.M., Williams, T., Bonfi, M., Dolecek, C., Hien, T.T., White, N.J., Michon, P., Siba, P., Teravang, L., Harf, G., Muller, I., White, N.J., M. K., Reeder, J.C., Beck, H.P., Picot, S., Genton, B., 2008. Molecular markers of in vivo drug sensitivity in Plasmodium falciparum and P. vivax. Nat. Genet. 41, 12–17.

Reeder, J.C., Beck, H.P., Picot, S., Genton, B., 2008. Molecular markers of in vivo drug sensitivity in Plasmodium falciparum and P. vivax. Nat. Genet. 41, 12–17.

Fairhurst, R.M., White, N.J., Kwiatkowski, D.P., 2015. Genetic architecture of Plasmodium falciparum and P. vivax. Antimicrob. Agents Chemother. 56, 5073–5078.
and sulfadoxine-pyrimethamine in southern Papua, Indonesia. Trans. R. Soc. Trop. Med. Hyg. 101, 351–359.

Richards, W.H., Williams, S.G., 1973. The removal of leucocytes from malaria infected blood. Ann. Trop. Med. Parasitol. 67, 249–250.

Riegel, B., Roepe, P.D., 2020. Altered drug transport by plasmodium falciparum chloroquine resistance transporter isoforms harboring mutations associated with piperazine resistance. Biochemistry 59, 2484–2495.

Ross, L.S., Dhingra, S.K., Mok, S., Yeo, T., Wicht, J.K., Kämpfornin, T., Takala-Harrison, S., Witkowski, B., Fairhurst, R.M., Ariey, F., Menard, D., Fidock, D.A., 2018. Emerging Southeast Asian PfCRT mutations confer Plasmodium falciparum resistance to the first-line antimalarial piperazine. Nat. Commun. 9, 3314.

Russell, B., Chalfein, F., Prasetyorini, B., Kenangalem, E., Pira, K., Suwanarusk, R., Brockman, A., Prayoga, P., Sugito, P., Cheng, Q., Tjitra, E., Anstey, N.M., Price, R.N., 2008. Determinants of in vitro drug susceptibility testing of Plasmodium vivax. Antimicrob. Agents Chemother. 52, 1040–1045.

Sa, J.M., Yamamoto, M.M., Fernandez-Becerra, C., de Azevedo, M.F., Papakrivos, J., Naude, B., Wellem, T.E., Del Portillo, H.A., 2006. Expression and function of pvcrt-o, a Plasmodium vivax ortholog of pfcrt. In: Plasmodium Falciparum and Dicystostelium discoideum. Molecular and Biochemical Parasitology, vol. 150, pp. 219–228.

Saunders, D.L., Vanachayangkul, P., Lon, C., Program, U.S.A.M.M.R., National Center for Parasitology, E., Malaria, C., Royal Cambodian Armed, F., 2014. Pyrazoleamide compounds are potent antimalarials that target Na+ homeostasis in intraerythrocytic Plasmodium falciparum. Nat. Commun. 5, 5521.

van der Pluijm, R.W., Imwong, M., Chau, N.H., Hoa, N.T., Thuy-Nhien, N.T., Vaidya, A.B., Morrisey, J.M., Zhang, Z., Das, S., Daly, T.M., Otto, T.D., Spillman, N.J., Trager, W., Jensen, J.B., 1976. Human malaria parasites in continuous culture. Science 193, 673–675.

Vaidya, A.B., Morrissiey, J.M., Zhang, Z., Das, S., Daly, T.M., Otto, T.D., Spillman, N.J., Wyvart, M., Siegl, P., Marfurt, J., Wirjanata, G., Sebayang, B.F., Price, R.N., Chatterjee, A., Nagle, A., Stasiak, M., Charmain, S.A., Angulo-Barturen, J., Ferrer, S., Belen Jimenez-Diaz, M., Martinez, M.S., Gamo, F.J., Avery, V.J., Ruerker, A., Delves, M., Kirk, K., Berriman, M., Kongtare, S., Burrows, J., Fan, E., Bergman, L.W., 2014. Pyrazoleamide compounds are potent antimarial targets that signal Na+ homeostasis in intraerythrocytic Plasmodium falciparum. Nat. Commun. 5, 5521.

van der Pluijm, R.W., Imwong, M., Chau, N.H., Hoa, N.T., Thuy-Nhien, N.T., Thanh, N.V., Jittamala, P., Harboonkunupakarn, B., Chutasm, K., Saelow, C., Runjarern, R., Kaewmokw, W., Tripura, R., Petto, T.D., Yoy, S., Suon, S., Streng, S., Mao, S., Oun, S., Yen, S., Amarutunga, C., Lek, D., Huy, R., Dhorda, M., Chotivanich, K., Ashley, E.A., Mukaka, M., Waithira, N., Chau, N.H., Price, R.N., Amato, R., Pearson, R.D., Moito, O., White, N.J., Donorop, A.M., 2019. Determinants of dihydroartemisinin-piperazine treatment failure in Plasmodium falciparum malaria in Cambodia, Thailand, and Vietnam: a prospective clinical, pharmacological, and genetic study. Lancet Infect. Dis. 19, 952–961.

Veiga, M.I., Dhingra, S.K., Henrich, P.P., Strainer, J., Gnadig, N., Uhlmann, A.C., Martin, R.E., Lehtane, A.M., Fidock, D.A., 2016. Globally prevalent PfMDR1 mutations modulate Plasmodium falciparum susceptibility to artemisinin-based combination therapies. Nat. Commun. 7, 11553.

Wirjanata, G., Sebayang, B.F., Chalfein, F., Prayoga, Handayuni, I., Noviyanti, R., Kenangalem, E., Poespoprodjo, J.R., Beggan, J., Price, R.N., Marfurt, J., 2015a. Contrasting ex vivo efficacies of “reversed chloroquine” compounds in chloroquine-resistant Plasmodium falciparum and P. vivax isolates. Antimicrob. Agents Chemother. 59, 5721–5726.

Wirjanata, G., Sebayang, B.F., Chalfein, F., Prayoga, Handayuni, I., Trianty, L., Kenangalem, E., Noviyanti, R., Campo, B., Poespoprodjo, J.R., Mohle, J.R., Price, N.R., Marfurt, J., 2015b. Potent ex vivo activity of naphthoquinone and methylene blue against drug-resistant clinical isolates of Plasmodium falciparum and plasmodium vivax. Antimicrob. Agents Chemother. 59, 6117–6124.

Wirjanata, G., Handayuni, I., Prayoga, P., Leonardo, L., Apriyanti, D., Trianty, L., Wandoe, R., Gobay, B., Kenangalem, E., Poespoprodjo, J.R., Noviyanti, R., Kyle, D.E., Cheng, Q., Price, R.N., Marfurt, J., 2017. Plasmodium falciparum and P. vivax demonstrate contrasting chloroquine resistance reversal phenotypes. Antimicrob. Agents Chemother. 61 (8), e00355-17 https://doi.org/10.1128/AAC.00355-17.

Witkowski, B., Duro, V., Khim, N., Ross, L.S., Saintpierre, B., Beghain, J., Chien, S., Kim, S., Ke, S., Koeung, N., Eam, R., Khean, C., Men, L., Koch, K., Rouillon, A., Domergue, A., Ma, L., Bouchier, C., Leang, R., Huy, R., Noel, G., Barale, J.C., Legrand, E., Ringwald, P., Fidock, D.A., Mercereau-Puijalon, O., Ariey, F., Menard, D., 2017. A surrogate marker of piperazine-resistant Plasmodium falciparum malaria: a phenotype-genotype association study. Lancet Infect. Dis. 17, 184–183.

Wongrigananala, C., Pickard, A.L., Wernsdorfer, W.H., Meshnick, S.R., 2002. Epidemiology of drug-resistant malaria. Lancet Infect. Dis. 2, 209–218.

Woodrow, C.J., Dahlstrom, S., Cooksey, R., Flegg, J.A., Le, N.H., Mentre, F., Murillo, C., Menard, D., Nosten, F., Siriprawat, K., Muesse, L., Quashie, N.B., Fairhurst, R.M., Edstein, M.D., Avery, V.M., Baell, J.B., 2019. 3,3'-Disubstituted 5,5'-Bi(1,2,4-triazine) derivatives with potent in vitro and in vivo antimalarial activity. J. Med. Chem. 62, 2485–2498.

Xue, L., Shi, D.H., Harjani, J.R., Huang, F., Beveridge, J.G., Dingjan, T., Ban, K., Dürb, S., Duffy, L., Lucantoni, L., Fletcher, S., Chau, F.K.C., Blundell, S., Ellis, K., Ralph, S.A., Marfurt, J., Charnan, S.A., Cuellar, M.E., Strasser, J.M., Dahlen, J.L., Walters, M.A., Edstein, M.D., Aver, V.M., Baell, J.B., 2019. 5,5'-Disubstituted 5,5'-Bi(1,2,4-triazine) derivatives with potent in vitro and in vivo antimalarial activity. J. Med. Chem. 62, 2485–2498.