AAC Journals.ASM.org

Contrasting *Ex Vivo* Efficacies of "Reversed Chloroquine" Compounds in Chloroquine-Resistant *Plasmodium falciparum* and *P. vivax* Isolates

Grennady Wirjanata,[a] Boni F. Sebayang,[b] Ferryanto Chalfein,[c] Prayoga,[c] Irene Handayuni,[a] Rintis Noviyanti,[b] Enny Kenangalem,[c,d] Jeanne Rini Poespoprodjo,[c,d,e] Steven J. Burgess,[f,g] David H. Peyton,[f,g] Ric N. Price,[a,h] Jutta Marfurt[a]

Global and Tropical Health Division, Menzies School of Health Research, Charles Darwin University, Casuarina, Darwin, Australia[a]; Eijkman Institute for Molecular Biology, Jakarta, Indonesia[b]; Papuan Health and Community Development Foundation (PHCDF), Timika, Papua, Indonesia[c]; District Health Authority, Timika, Papua, Indonesia[d]; Department of Paediatrics, Faculty of Medicine, Gadjah Mada University, Yogyakarta, Indonesia[e]; Department of Chemistry, Portland State University, Portland, Oregon, USA[f]; DesignMedix, Inc., Portland, Oregon, USA[g]; Centre for Tropical Medicine and Global Health, Nuffield Department of Clinical Medicine, University of Oxford, Oxford, United Kingdom[h]

Chloroquine (CQ) has been the mainstay of malaria treatment for more than 60 years. However, the emergence and spread of CQ resistance now restrict its use to only a few areas where malaria is endemic. The aim of the present study was to investigate whether a novel combination of a CQ-like moiety and an imipramine-like pharmacophore can reverse CQ resistance *ex vivo*. Between March to October 2011 and January to September 2013, two "reversed chloroquine" (RCQ) compounds (PL69 and PL106) were tested against multidrug-resistant field isolates of *Plasmodium falciparum* ($n = 41$) and *Plasmodium vivax* ($n = 45$) in Papua, Indonesia, using a modified *ex vivo* schizont maturation assay. The RCQ compounds showed high efficacy against both CQ-resistant *P. falciparum* and *P. vivax* field isolates. For *P. falciparum*, the median 50% inhibitory concentrations (IC_{50}s) were 23.2 nM for PL69 and 26.6 nM for PL106, compared to 79.4 nM for unmodified CQ ($P < 0.001$ and $P = 0.036$, respectively). The corresponding values for *P. vivax* were 19.0, 60.0, and 60.9 nM ($P < 0.001$ and $P = 0.018$, respectively). There was a significant correlation between IC_{50}s of CQ and PL69 (Spearman's rank correlation coefficient [r_s] = 0.727, $P < 0.001$) and PL106 ($r_s = 0.830$, $P < 0.001$) in *P. vivax* but not in *P. falciparum*. Both RCQs were equally active against the ring and trophozoite stages of *P. falciparum*, but in *P. vivax*, PL69 and PL106 showed less potent activity against trophozoite stages (median IC_{50}s, 130.2 and 172.5 nM) compared to ring stages (median IC_{50}s, 17.6 and 91.3 nM). RCQ compounds have enhanced *ex vivo* activity against CQ-resistant clinical isolates of *P. falciparum* and *P. vivax*, suggesting the potential use of reversal agents in antimalarial drug development. Interspecies differences in RCQ compound activity may indicate differences in CQ pharmacokinetics between the two *Plasmodium* species.

Malaria remains one of the most important infectious diseases in the world (1). A key component of malaria control programs is the ability to provide early diagnosis and treatment with effective antimalarial drugs. Chloroquine (CQ) has been the most commonly used drug for the treatment and prevention of malaria since the 1940s; it is cheap, well tolerated, and widely available. However, within a decade of its deployment, the first reports of CQ resistance were noted in Southeast Asia and South America. By the 1980s, CQ was no longer effective in much of these regions, and reports began to emerge of declining efficacy in Africa (2). It is estimated that more than 80% of the world's malaria is now resistant to CQ (3), leading the World Health Organization (WHO) to recommend that the use of CQ be restricted to non-*falciparum* malarias and the treatment of *falciparum* malaria in a few specific areas only (4).

CQ resistance in *P. falciparum* is a multigenic process (5), the main molecular determinant being mutations in the gene encoding PfCRT (*Plasmodium falciparum* chloroquine resistance transporter), a putative channel involved in enhanced CQ efflux from the parasite's digestive vacuole (DV) (6).

Several resistance reversal agents, also known as chemosensitizers, have been identified and shown to inhibit CQ efflux in CQ-resistant parasites (7). Many of these agents are licensed for alternative medical conditions, such as antidepressants (desipramine), antihistamines (chlorpheniramine), and calcium channel blockers (verapamil). However, the doses required to achieve adequate "reversal activity" *in vivo* can lead to poor tolerability and adverse side effects for the patient (8).

One of the strategies to improve both pharmacokinetic and pharmacodynamic properties of CQ resistance reversal agents is to combine the reversal agent with CQ (8, 9). In 2006, Burgess et al. reported innovative hybrid compounds, "reversed CQ" (RCQ) molecules, combining a CQ-like moiety with a CQ resistance reversal agent closely related to imipramine (10). *In vivo* drug susceptibility testing showed that these molecules are potent *in vitro* growth inhibitors of CQ-susceptible and CQ-resistant *P. falciparum* laboratory strains, with 50% inhibitory concentrations (IC_{50}s) in the low-nanomolar range (10). Subsequent studies in animals have confirmed antimalarial efficacy with no overt signs of toxicity (11). These compounds result in enhanced uptake and accumulation in the parasite's digestive vacuole of CQ-resistant parasites and inhibition of hemozoin production in a manner

Received 4 May 2015 **Returned for modification** 30 May 2015
Accepted 30 June 2015
Accepted manuscript posted online 6 July 2015
Citation Wirjanata G, Sebayang BF, Chalfein F, Prayoga, Handayuni I, Noviyanti R, Kenangalem E, Poespoprodjo JR, Burgess SJ, Peyton DH, Price RN, Marfurt J. 2015. Contrasting *ex vivo* efficacies of "reversed chloroquine" compounds in chloroquine-resistant *Plasmodium falciparum* and *P. vivax* isolates. Antimicrob Agents Chemother 59:5721–5726. doi:10.1128/AAC.01048-15.
Address correspondence to Jutta Marfurt, jutta.marfurt@menzies.edu.au.

doi:10.1128/AAC.01048-15

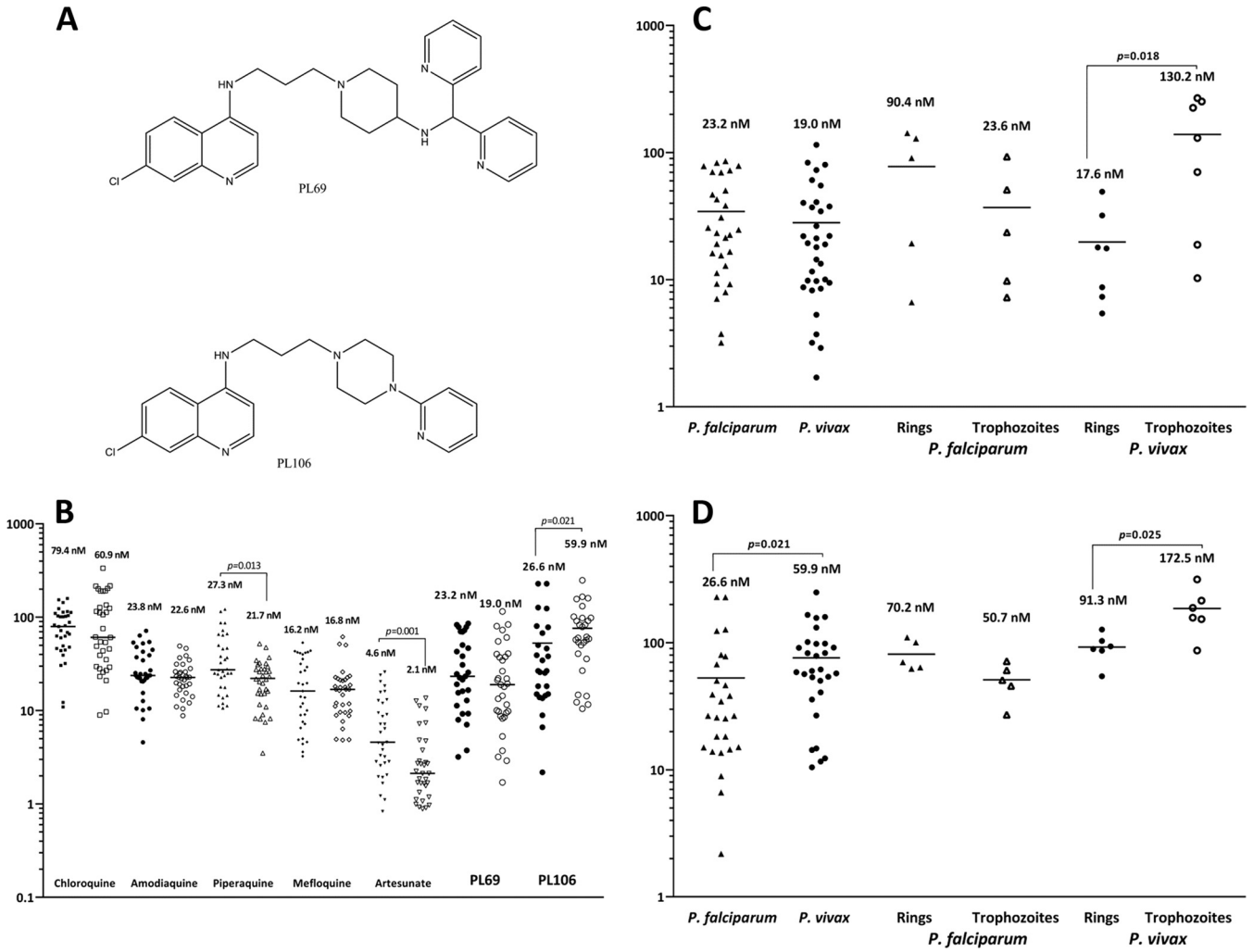

FIG 1 (A) Chemical structure of "reversed chloroquine" (RCQ) compounds PL69 (compound 22 in reference 11) and PL106. (B) *Ex vivo* drug susceptibility (median IC_{50}s) to standard antimalarials and the RCQ compounds PL69 and PL106 in *P. falciparum* (closed symbols) and *P. vivax* (open symbols) clinical isolates. Significance of *P* values was determined by Wilcoxon rank sum test. (C and D) *Ex vivo* drug susceptibilities (median IC_{50}s) for PL69 (C) and PL106 (D) according to species tested and for paired ring-stage (closed symbols) versus trophozoite-stage (open symbols) parasites. Significance of *P* values was determined by Wilcoxon rank sum test.

analogous to, but more potent than, that of CQ (11). However, the efficacy of RCQs against *P. falciparum* field isolates has not been reported, and there are no data on the efficacy of RCQ compounds against *P. vivax*.

The aims of this study were to (i) investigate the *ex vivo* susceptibility profiles of two RCQ compounds, PL69 and PL106 (Fig. 1A), against drug-resistant clinical isolates of *P. falciparum* and *P. vivax* in Papua, Indonesia, (ii) to examine the stage-specific action of these compounds, and (iii) to investigate cross-susceptibility patterns with conventional antimalarials.

MATERIALS AND METHODS

Study area and field sample collection. The studies were conducted in Timika, Papua Province, Indonesia, between March and October in 2011 and January and September in 2013. Patients presenting to the Rumah Sakit Mitra Masyarakat (RSMM) Hospital were enrolled into the study if diagnosed with a microscopically confirmed *P. falciparum* or *Plasmodium vivax* monoinfection and a parasitemia of between 2,000 and 80,000 parasites/μl. Isolates of *Plasmodium* spp. were processed only if at least 70% of asexual forms were at the ring stage (12). Patients treated with CQ in the last 30 days or any other antimalarial treatments in the last 2 weeks or hemoglobin levels below 5 g/dl were excluded. With written informed consent, 5 ml of venous blood was collected by venipuncture and processed immediately. Host white blood cells were removed using cellulose columns as previously described (13), and packed infected red blood cells (iRBC) were used for the *ex vivo* drug susceptibility assay.

Drug compounds and drug plate preparation. Each drug plate (96 wells) contained 11 serial concentrations (2-fold dilutions) in duplicate of four antimalarials. The following antimalarial drugs were assayed: chloroquine (CQ), amodiaquine (AQ), piperaquine (PIP), mefloquine (MFQ), and artesunate (AS) (all provided by the WWARN QA/QC Reference Material Programme [14]), as well as the "reversed CQ" (RCQ) compounds PL69 and PL106 (DesignMedix, Inc., Portland, OR) (Fig. 1A). Predosed drug plates were made by adding 25 μl of drug dilutions to each well, followed by lyophilization and storage at 4°C. The following maximum concentrations were used: 2,993 nM for CQ, 158 nM for AQ, 1,029 for PIP, 338 nM for MFQ, 49 nM for AS, and 295 nM and 349 nM for the RCQ compounds PL69 and PL106, respectively.

TABLE 1 Baseline characteristics of isolates for which *ex vivo* assays were accomplished

Baseline characteristic	*P. falciparum* ($n = 41$)	*P. vivax* ($n = 45$)
Isolates reaching harvest, no. (%)	33 (80.5)	34 (75.6)
Median delay from venipuncture to start of culture, min (range)	153 (85–280)	180 (100–330)
Median duration of assay, h (range)	46 (30–49)	48 (43–50)
Geometric mean parasitemia, no. of asexual parasites/μl (95% CI)[a]	29,342 (19,286–44,639)	20,936 (14,293–30,666)
Median initial amt of parasites at ring stage, % (range)	100[b]	95 (71–99)
Mean schizont count at harvest, % (95% CI)	46 (16–76)	43 (25–70)

[a] CI, confidence interval.
[b] No range given. (All values were 100%.)

***Ex vivo* drug susceptibility assay.** Drug susceptibilities of *Plasmodium* isolates were measured using a modified schizont maturation assay as described previously (15, 16). Drug susceptibility profiles were presented as inhibition of parasite growth from the ring to schizont stage. Two hundred microliters of a 2% hematocrit blood medium mixture (BMM), consisting of RPMI 1640 medium supplemented with 10% AB^+ human serum (for *P. falciparum*) or McCoy's 5A medium supplemented with 20% AB^+ human serum (for *P. vivax*), was added to each well of the predosed 96-well drug plates. The parasites were cultured in a candle jar at 37.0°C for 34 to 50 h. Incubation was stopped when more than 40% of ring-stage parasites had reached the mature schizont stage in the drug-free control well.

The plates were harvested by preparing thick blood films from each well of the plates. Thick blood films were stained with 5% Giemsa solution for 25 min and examined microscopically. Differential counts of 200 asexual parasites in the test slides were classified into ring stages, trophozoites, and mature schizonts. To reduce parasite classification error and ease parasite identification, only schizonts with at least 5 or more well-defined chromatin dots were classified as schizonts at harvest. Free merozoites and gametocytes were not included in the count. To determine the effect of each antimalarial drug, the number of schizonts was determined for each drug concentration and then normalized to that of the corresponding drug-free control well.

To investigate the stage-specific drug susceptibility, *Plasmodium* isolates with greater than 90% rings were set up (i) in culture in the presence of drug directly for 24 h and after washing out of the drug for another 20 to 24 h and (ii) after culture in the absence of drug for 20 to 24 h to achieve 90% trophozoites that were then drug exposed for 20 to 24 h (17).

Data analysis. The raw dose-response data were analyzed using nonlinear regression analysis (WinNonLn 4.1; Pharsight Corporation), and the IC_{50}s were derived using an inhibitory sigmoid E_{max} model. *Ex vivo* IC_{50} data were used only from predicted curves where the E_{max} (maximum effect) and E_0 (minimum effect) were within 15% of 100 or 0, respectively. Data analysis was performed using the STATA (version 13.1; Stata Corp., College Station, TX) and GraphPad Prism (version 6) software packages. The Mann-Whitney U test, Wilcoxon signed-rank test, and Spearman's rank correlation were used for nonparametric comparisons and correlations.

Ethical approval. Ethical approval for this project was obtained from the Human Research Ethics Committee of the NT Department of Health & Families and Menzies School of Health Research, Darwin, Australia (HREC 2010-1396) and the Eijkman Institute Research Ethics Commission, Jakarta, Indonesia (EIREC-47) in September 2010.

RESULTS

Antimalarial susceptibility. *Ex vivo* drug susceptibility profiles were attempted in 86 clinical isolates; baseline characteristics of these isolates are presented in Table 1. Adequate growth for harvest was achieved in 81% (33/41) of *P. falciparum* isolates and 76% (34/45) of *P. vivax* isolates. The median IC_{50}s for all clinical isolates successfully cultured are shown in in Fig. 1B and Table 2. PL69 IC_{50} estimates could not be derived for four *P. falciparum* isolates (MIC, >295 nM) and one *P. vivax* isolate (MIC, >349 nM).

Drug susceptibility differed significantly between species, with IC_{50}s being higher in *P. falciparum* than in *P. vivax* for PIP (median IC_{50} = 27.3 nM versus 21.7 nM; $P = 0.012$) and artesunate (median IC_{50} = 4.6 nM versus 2.1 nM; $P = 0.001$) but lower for PL106 (median = 26.6 versus 59.9 nM; $P = 0.021$). In *P. falciparum* isolates, IC_{50}s were significantly lower for PL69 and PL106 than for CQ. In *P. vivax*, IC_{50}s for PL69 (median = 19.0 nM; $P <$

TABLE 2 Overall *ex vivo* susceptibility for each drug according to the species tested

Drug	*P. falciparum*				*P. vivax* clinical field isolates		
	Laboratory line (IC_{50}, nM)[a]		Clinical field isolates				
	FC27 (CQ^s)	K1 (CQ^r)	No. of assays[b]	Median IC_{50}, nM (range)	No. of assays[b]	Median IC_{50}, nM (range)	P[c]
Chloroquine	24.09	140.48	33	79.36 (10.92–158.66)	34	60.86 (8.96–334.55)	0.990
Amodiaquine	20.27	26.89	33	23.76 (4.56–71.38)	33	22.63 (8.80–49.16)	0.211
Piperaquine	31.72	47.21	33	27.30 (10.62–121.16)	34	21.69 (3.50–51.86)	0.013
Mefloquine	53.77	13.74	33	16.16 (3.23–53.29)	34	16.82 (4.83–61.48)	0.082
Artesunate	10.12	7.70	33	4.58 (0.83–25.90)	34	2.12 (0.88–13.54)	0.001
PL69	28.49	41.72	29	23.24 (3.18–85.54)	33	18.95 (1.70–114.87)	0.250
PL106	52.56	47.87	27	26.55 (2.18–228.26)	29	59.89 (10.45–248.41)	0.021

[a] Mean IC_{50}s (derived from 3 independent experiments) were assessed by *in vitro* schizont maturation quantified by microscopy. CQ^s, chloroquine-sensitive laboratory strain; CQ^r, chloroquine-resistant laboratory strain.
[b] Total number of assays with acceptable drug assay results.
[c] Significant difference in median drug IC_{50} between species.

TABLE 3 *Ex vivo* susceptibilities for paired isolates tested with 24 h of drug exposure at ring and trophozoite stage

Drug	*P. falciparum*				*P. vivax*			
		Median IC_{50}, nM (range)				Median IC_{50}, nM (range)		
	n^a	Rings	Trophozoites	P^b	n^a	Rings	Trophozoites	P^b
Chloroquine	5	107.87 (60.17–163.58)	142.71 (25.50–163.64)	0.855	8	88.87 (41.85–190.17)	778.18 (99.88–4,033.31)	0.003
Amodiaquine	5	16.23 (4.86–201.77)	17.96 (7.76–25.43)	0.570	8	21.61 (5.69–39.47)	53.22 (7.57–89.76)	0.062
Piperaquine	5	21.85 (7.78–33.28)	128.19 (13.80–359.25)	0.044	8	20.88 (9.76–69.60)	30.34 (18.08–205.52)	0.076
Mefloquine	5	15.26 (4.74–46.88)	17.26 (4.60–30.56)	0.570	8	17.87 (8.17–41.00)	37.06 (11.88–85.49)	0.062
Artesunate	5	2.10 (0.74–5.11)	3.87 (1.28–8.13)	0.584	8	1.91 (1.21–4.57)	7.20 (0.83–19.32)	0.054
PL69	5	90.39 (6.61–142.06)	23.55 (7.25–92.82)	0.465	7	17.59 (5.42–49.13)	130.22 (10.26–268.15)	0.018
PL106	5	70.17 (62.24–110.05)	50.67 (27.06–71.36)	0.047	6	91.25 (54.35–126.84)	172.48 (86.76–314.27)	0.025

[a] *n*, number of paired isolates.
[b] Comparison of drugs tested at ring and trophozoite stage, respectively (Wilcoxon rank sum test).

0.001) but not PL106 (median = 59.9 nM) were significantly lower than those for CQ (median = 60.9 nM).

Stage-specific drug susceptibility. Stage-specific drug susceptibility was investigated in 5 *P. falciparum* and 7 *P. vivax* isolates (Table 3 and Fig. 1C and D). *P. falciparum* isolates assayed at trophozoite stage had higher IC_{50}s than with drug exposure at the ring stage for PIP (median = 128.2 nM versus 21.9 nM) but lower IC_{50}s for PL69 (median = 50.7 nM versus 70.2 nM), although this was of borderline significance ($P = 0.044$ and 0.047, respectively). In contrast, *P. vivax* isolates assayed at trophozoite stage had significantly higher IC_{50s} for CQ (median = 778.2 nM versus 88.9 nM; $P = 0.003$), AS (median = 7.2 nM versus 1.9 nM; $P = 0.054$), PL69 (median = 130.2 nM versus 17.6 nM; $P = 0.018$), and PL106 (median = 172.5 nM versus 91.3 nM; $P = 0.025$).

Cross-susceptibility patterns. Spearman's rank correlation coefficients (r_s) are presented in Table 4. PL69 showed significantly positive correlations in both *P. falciparum* and *P. vivax* for AQ ($r_s = 0.512$ and 0.457, respectively), PIP ($r_s = 0.631$ and 0.566), and MFQ ($r_s = 0.480$ and 0.556). However, the correlations of PL69 were only significant with AS in *P. falciparum* ($r_s = 0.446$, $P = 0.015$) and CQ in *P. vivax* ($r_s = 0.727$, $P < 0.001$). Contrasting correlation patterns between species were observed for PL106 with significant positive correlations in *P. falciparum* only apparent with PIP ($r_s = 0.456$, $P = 0.019$). In *P. vivax*, PL106 was positively correlated with CQ ($r_s = 0.830$, $P < 0.001$), AQ ($r_s = 0.505$, $P = 0.005$), and MFQ ($r_s = 0.401$, $P = 0.031$) and negatively correlated with AS ($r_s = -0.470$, $P = 0.010$).

TABLE 4 Correlation of *ex vivo* antimalarial susceptibilities in *P. falciparum* and *P. vivax* clinical field isolates

Antimalarial combination	*P. falciparum*			*P. vivax*		
	r_s^a	*P*	df[b]	r_s^a	*P*	df[b]
PL69-CQ	0.097	0.618	29	0.727	<0.001	33
PL69-AQ	0.512	0.005	29	0.457	0.008	33
PL69-PIP	0.631	<0.001	29	0.566	0.001	33
PL69-MFQ	0.480	0.008	29	0.556	0.001	33
PL69-AS	0.446	0.015	29	−0.124	0.492	33
PL106-CQ	0.113	0.582	26	0.830	<0.001	29
PL106-AQ	−0.036	0.862	26	0.505	0.005	29
PL106-PIP	0.456	0.019	26	0.332	0.079	29
PL106-MFQ	0.220	0.281	26	0.401	0.031	29
PL106-AS	−0.108	0.601	26	−0.470	0.010	29
PL106-PL69	0.472	0.023	23	0.694	<0.001	29

[a] r_s, Spearman rank correlation coefficient.
[b] df, degrees of freedom.

DISCUSSION

This is the first report of the *ex vivo* drug susceptibilities of two novel RCQ compounds, PL69 and PL106, against multidrug-resistant clinical field isolates of *P. falciparum* and *P. vivax*. The IC_{50}s of PL69 were 3-fold lower than those of CQ in both *P. falciparum* and *P. vivax* and similar to those of AQ and PIP in both species. While PL106 was also 3-fold more potent than CQ against *P. falciparum*, it was only moderately more potent than CQ in *P. vivax*. However, 5 isolates (four of *P. falciparum* and one of *P. vivax*) had elevated PL69 MICs, with the range of drug concentrations assessed precluding derivation of reliable IC_{50}s. The significance of these findings warrants further molecular analysis.

Using our schizont maturation assay, the RCQ IC_{50}s in the laboratory strains FC27 and K1 ranged from 28.5 to 41.7 nM for PL69 and 47.9 to 52.6 nM for PL106. These values are significantly higher than the unpublished data derived using a SYBR green I assay (range, 0.9 to 1.8 nM and 1.7 to 2.7 nM, respectively) (11). This may reflect differences in the assay methodology. Previous data for these RCQ compounds were derived by proliferation-based growth inhibition assays of culture-adapted laboratory strains in which *in vitro* growth was assessed by quantification of DNA using a SYBR green I fluorescence-based assay. This methodology generally produces similar results to radiotracer methods such as [^{3}H]hypoxanthine incorporation (18) but is known to produce significantly lower IC_{50}s than the field-based schizont maturation assay used in the present study (19, 20).

Although the IC_{50}s of the standard antimalarials were similar between species, the IC_{50}s of PL106 in *P. vivax* were almost 2-fold higher than those observed in *P. falciparum*. However, since both compounds (albeit PL106 to a lesser extent) have higher activity than CQ in isolates of both species, this suggests an overall lower CQ resistance reversal activity of PL106 than PL69 in *P. vivax*.

The two RCQ compounds showed notable differences in the correlation patterns between and within the two *Plasmodium* species (Table 4). Whereas statistically significant positive correlations were found between PL69 and the quinoline-based antimalarials AQ, PIP, and MFQ in both species, a strongly positive correlation with CQ was observed in *P. vivax* only ($r_s = 0.727$, $P < 0.001$). The same was observed with PL106, which showed a strong and significant positive correlation with CQ in *P. vivax* ($r_s = 0.830$, $P < 0.001$) but not in *P. falciparum*. This observation

most likely reflects the expected positive correlation between the CQ moiety of the RCQ compounds and CQ due to their shared modes of uptake, partition, and action; this action is abolished by the potent reversal activity of these compounds in CQ-resistant *P. falciparum* isolates. However, the fact that the same phenomenon was not observed in *P. vivax* isolates indicates again that both RCQ compounds have greater CQ resistance reversal potential in *P. falciparum* than in *P. vivax*. This is further corroborated by the strong and significant correlation that was observed between PL69 and PL106 in *P. vivax* ($r_s = 0.694$, $P < 0.001$) but not in *P. falciparum*.

Our results confirm previous observations demonstrating the marked stage specificity of chloroquine (12, 17) in *P. vivax*, with trophozoites being almost completely resistant to CQ. IC_{50}s for *P. vivax* trophozoite stages were also higher for AS, PL69, and PL106 than for the ring stages of the same isolates, but this was not apparent in *P. falciparum* (Table 3). Although *P. vivax* trophozoites showed significantly higher median IC_{50}s for PL69 (7-fold) and PL106 (2-fold) than the ring stages, the values of the RCQ compounds against *P. vivax* trophozoites were almost a log fold lower than those of CQ, suggesting that some resistance reversal activity was exerted against both stages.

Our results demonstrating *ex vivo* IC_{50}s of the RCQ compounds against CQ-resistant *P. falciparum* field isolates in the low- and mid-nanomolar ranges are in line with the data obtained with *P. falciparum* laboratory strains. A previous study of *P. vivax* isolates showed no significant difference between CQ IC_{50}s of isolates exposed to CQ alone compared to isolates exposed to CQ plus verapamil (21). In the present study, RCQ IC_{50}s in *P. vivax* were lower than those of CQ, which suggests that the resistance reverser moiety of the RCQ compounds does have an effect on CQ activity in *P. vivax* isolates. The mechanisms of both CQ resistance and CQ resistance reversal activity of various chemosensitizers have been investigated in *P. falciparum* and revealed *pfcrt* to be a key determinant of resistance and a target for chemosensitizers; however, both processes are likely to be mediated by multiple factors (5, 22, 23). The knowledge of these processes in *P. vivax* is still elusive. By using heterologous expression systems, Sa and colleagues have shown that the *pfcrt* orthologue *pvcrt-o* plays a role in CQ accumulation and that increased accumulation could be reverted by verapamil (24). Our data demonstrating the CQ chemosensitizing effect for the imipramine-like reversal agent in *P. vivax* suggests that CQ resistance in this species may also be mediated by decreased drug accumulation; however, further investigations are needed to better understand CQ pharmacokinetics and its genetic determinants in *P. vivax*.

The RCQ compounds demonstrate significant enhancement of *ex vivo* activity over CQ against CQ-sensitive and CQ-resistant clinical isolates of both *P. falciparum* and *P. vivax* and highlight the potential approach of reversal agents in antimalarial drug development. Although the action of the RCQ compounds against *P. vivax* has yet to be elucidated further, the current variation of RCQ compounds between *P. falciparum* and *P. vivax* supports a growing body of work suggesting that CQ uptake and metabolism, as well as CQ resistance mechanisms, in these two species may be different (25).

ACKNOWLEDGMENTS

We are grateful to Lembaga Pengembangan Masyarakat Amungme Kamoro, the staff of the Rumah Sakit Mitra Masyarakat (RSMM) Hospital, and Paulus Sugiarto for their support in conducting this study. We thank the Australian Red Cross blood transfusion service for the supply of human sera.

The study was funded by the Wellcome Trust (Senior Research Fellowship in Clinical Science 091625 to R.N.P. and Research Training Fellowship 099875 to J.R.P.), the National Health and Medical Research Council (Project Grant 1023438 to R.N.P. and J.M. and Program Grant 1037304 to R.N.P.), and the Swiss National Science Foundation (Fellowship for Advanced Researchers to J.M.).

REFERENCES

1. **World Health Organization.** 2014. World malaria report 2014. World Health Organization, Geneva, Switzerland.
2. **Wellems TE, Plowe CV.** 2001. Chloroquine-resistant malaria. J Infect Dis **184:**770–776. http://dx.doi.org/10.1086/322858.
3. **Talisuna AO, Bloland P, D'Alessandro U.** 2004. History, dynamics, and public health importance of malaria parasite resistance. Clin Microbiol Rev **17:**235–254. http://dx.doi.org/10.1128/CMR.17.1.235-254.2004.
4. **World Health Organization.** 2010. Guidelines for the treatment of malaria, 2nd ed, 2014 ed. World Health Organization, Geneva, Switzerland.
5. **Mu J, Ferdig MT, Feng X, Joy DA, Duan J, Furuya T, Subramanian G, Aravind L, Cooper RA, Wootton JC, Xiong M, Su XZ.** 2003. Multiple transporters associated with malaria parasite responses to chloroquine and quinine. Mol Microbiol **49:**977–989. http://dx.doi.org/10.1046/j.1365-2958.2003.03627.x.
6. **Martin RE, Marchetti RV, Cowan AI, Howitt SM, Broer S, Kirk K.** 2009. Chloroquine transport via the malaria parasite's chloroquine resistance transporter. Science **325:**1680–1682. http://dx.doi.org/10.1126/science.1175667.
7. **Guantai E, Chibale K.** 2010. Chloroquine resistance: proposed mechanisms and countermeasures. Curr Drug Deliv **7:**312–323. http://dx.doi.org/10.2174/156720110793360577.
8. **van Schalkwyk DA, Egan TJ.** 2006. Quinoline-resistance reversing agents for the malaria parasite *Plasmodium falciparum*. Drug Resist Updat **9:**211–226.
9. **Egan TJ, Kaschula CH.** 2007. Strategies to reverse drug resistance in malaria. Curr Opin Infect Dis **20:**598–604. http://dx.doi.org/10.1097/QCO.0b013e3282f1673a.
10. **Burgess SJ, Selzer A, Kelly JX, Smilkstein MJ, Riscoe MK, Peyton DH.** 2006. A chloroquine-like molecule designed to reverse resistance in *Plasmodium falciparum*. J Med Chem **49:**5623–5625. http://dx.doi.org/10.1021/jm060399n.
11. **Burgess SJ, Kelly JX, Shomloo S, Wittlin S, Brun R, Liebmann K, Peyton DH.** 2010. Synthesis, structure-activity relationship, and mode-of-action studies of antimalarial reversed chloroquine compounds. J Med Chem **53:**6477–6489. http://dx.doi.org/10.1021/jm1006484.
12. **Russell B, Chalfein F, Prasetyorini B, Kenangalem E, Piera K, Suwanarusk R, Brockman A, Prayoga P, Sugiarto P, Cheng Q, Tjitra E, Anstey NM, Price RN.** 2008. Determinants of in vitro drug susceptibility testing of *Plasmodium vivax*. Antimicrob Agents Chemother **52:**1040–1045. http://dx.doi.org/10.1128/AAC.01334-07.
13. **Sriprawat K, Kaewpongsri S, Suwanarusk R, Leimanis ML, Lek-Uthai U, Phyo AP, Snounou G, Russell B, Renia L, Nosten F.** 2009. Effective and cheap removal of leukocytes and platelets from *Plasmodium vivax* infected blood. Malar J **8:**115. http://dx.doi.org/10.1186/1475-2875-8-115.
14. **Lourens C, Watkins WM, Barnes KI, Sibley CH, Guerin PJ, White NJ, Lindegardh N.** 2010. Implementation of a reference standard and proficiency testing programme by the World Wide Antimalarial Resistance Network (WWARN). Malar J **9:**375. http://dx.doi.org/10.1186/1475-2875-9-375.
15. **Marfurt J, Chalfein F, Prayoga P, Wabiser F, Kenangalem E, Piera KA, Machunter B, Tjitra E, Anstey NM, Price RN.** 2011. Ex vivo drug susceptibility of ferroquine against chloroquine-resistant isolates of *Plasmodium falciparum* and *P. vivax*. Antimicrob Agents Chemother **55:**4461–4464. http://dx.doi.org/10.1128/AAC.01375-10.
16. **Marfurt J, Chalfein F, Prayoga P, Wabiser F, Wirjanata G, Sebayang B, Piera KA, Wittlin S, Haynes RK, Mohrle JJ, Anstey NM, Kenangalem E, Price RN.** 2012. Comparative ex vivo activity of novel endoperoxides in multidrug-resistant *Plasmodium falciparum* and *P. vivax*. Antimicrob Agents Chemother **56:**5258–5263. http://dx.doi.org/10.1128/AAC.00283-12.
17. **Sharrock WW, Suwanarusk R, Lek-Uthai U, Edstein MD, Kosaisavee V, Travers T, Jaidee A, Sriprawat K, Price RN, Nosten F, Russell B.** 2008.

Plasmodium vivax trophozoites insensitive to chloroquine. Malar J **7:**94. http://dx.doi.org/10.1186/1475-2875-7-94.

18. **Smilkstein M, Sriwilaijaroen N, Kelly JX, Wilairat P, Riscoe M.** 2004. Simple and inexpensive fluorescence-based technique for high-throughput antimalarial drug screening. Antimicrob Agents Chemother **48:**1803–1806. http://dx.doi.org/10.1128/AAC.48.5.1803-1806.2004.
19. **Kocken CH, van der Wel A, Arbe-Barnes S, Brun R, Matile H, Scheurer C, Wittlin S, Thomas AW.** 2006. *Plasmodium vivax*: in vitro susceptibility of blood stages to synthetic trioxolane compounds and the diamidine DB75. Exp Parasitol **113:**197–200. http://dx.doi.org/10.1016/j.exppara.2005.12.016.
20. **Sisowath C, Petersen I, Veiga MI, Martensson A, Premji Z, Bjorkman A, Fidock DA, Gil JP.** 2009. In vivo selection of *Plasmodium falciparum* parasites carrying the chloroquine-susceptible pfcrt K76 allele after treatment with artemether-lumefantrine in Africa. J Infect Dis **199:**750–757. http://dx.doi.org/10.1086/596738.
21. **Suwanarusk R, Russell B, Chavchich M, Chalfein F, Kenangalem E, Kosaisavee V, Prasetyorini B, Piera KA, Barends M, Brockman A, Lek-Uthai U, Anstey NM, Tjitra E, Nosten F, Cheng Q, Price RN.** 2007. Chloroquine resistant *Plasmodium vivax*: in vitro characterisation and association with molecular polymorphisms. PLoS One **2:**e1089. http://dx.doi.org/10.1371/journal.pone.0001089.
22. **Patel JJ, Thacker D, Tan JC, Pleeter P, Checkley L, Gonzales JM, Deng B, Roepe PD, Cooper RA, Ferdig MT.** 2010. Chloroquine susceptibility and reversibility in a *Plasmodium falciparum* genetic cross. Mol Microbiol **78:**770–787. http://dx.doi.org/10.1111/j.1365-2958.2010.07366.x.
23. **Zishiri VK, Joshi MC, Hunter R, Chibale K, Smith PJ, Summers RL, Martin RE, Egan TJ.** 2011. Quinoline antimalarials containing a dibemethin group are active against chloroquinone-resistant *Plasmodium falciparum* and inhibit chloroquine transport via the *P. falciparum* chloroquine-resistance transporter (PfCRT). J Med Chem **54:**6956–6968. http://dx.doi.org/10.1021/jm2009698.
24. **Sa JM, Nomura T, Neves J, Baird JK, Wellems TE, del Portillo HA.** 2005. *Plasmodium vivax*: allele variants of the mdr1 gene do not associate with chloroquine resistance among isolates from Brazil, Papua, and monkey-adapted strains. Exp Parasitol **109:**256–259. http://dx.doi.org/10.1016/j.exppara.2004.12.005.
25. **Nomura T, Carlton JM, Baird JK, del Portillo HA, Fryauff DJ, Rathore D, Fidock DA, Su X, Collins WE, McCutchan TF, Wootton JC, Wellems TE.** 2001. Evidence for different mechanisms of chloroquine resistance in 2 *Plasmodium* species that cause human malaria. J Infect Dis **183:**1653–1661. http://dx.doi.org/10.1086/320707.