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In Vitro Activity of Pyronaridine against Multidrug-Resistant *Plasmodium falciparum* and *Plasmodium vivax*▽

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Pyronaridine, a Mannich base antimalarial, has demonstrated high *in vivo* and *in vitro* efficacy against chloroquine-resistant *Plasmodium falciparum*. Although this drug has the potential to become a prominent artemisinin combination therapy, little is known about its efficacy against drug-resistant *Plasmodium vivax*. The *in vitro* antimalarial susceptibility of pyronaridine was assessed in multidrug-resistant *P. vivax* ($n = 99$) and *P. falciparum* ($n = 90$) isolates from Papua, Indonesia, using a schizont maturation assay. The median 50% inhibitory concentration (IC_{50}) of pyronaridine was 1.92 nM (range, 0.24 to 13.8 nM) against *P. falciparum* and 2.58 nM (range, 0.13 to 43.6 nM) against *P. vivax*, with *in vitro* susceptibility correlating significantly with chloroquine, amodiaquine, and piperaquine (r_s [Spearman's rank correlation coefficient] = 0.45 to 0.62; $P < 0.001$). *P. falciparum* parasites initially at trophozoite stage had higher IC_{50}s of pyronaridine than those exposed at the ring stage (8.9 nM [range, 0.6 to 8.9 nM] versus 1.6 nM [range, 0.6 to 8.9 nM], respectively; $P = 0.015$), although this did not reach significance for *P. vivax* (4.7 nM [range, 1.4 to 18.7 nM] versus 2.5 nM [range, 1.4 to 15.6 nM], respectively; $P = 0.085$). The excellent *in vitro* efficacy of pyronaridine against both chloroquine-resistant *P. vivax* and *P. falciparum* highlights the suitability of the drug as a novel partner for artemisinin-based combination therapy in regions where the two species are coendemic.

Almost 40% of the world's population is at risk for infection by *Plasmodium vivax*, with an estimated 132 to 391 million clinical infections each year (19). Although chloroquine (CQ) remains the treatment of choice in most of the *P. vivax*-endemic world, this status is now being undermined by the emergence and spread of chloroquine-resistant (CQR) *P. vivax*. First reported in the 1980s on the island of New Guinea (2, 23), CQR *P. vivax* has since spread to other parts of Asia and recently to South America (1). In Papua, Indonesia, CQ resistance in *P. vivax* has reached levels precluding the use of CQ in most of the province (22, 30). There is an urgency to assess the efficacies of alternative antimalarial agents against drug-resistant *P. vivax* and to develop new strategies to combat the parasite.

Pyronaridine (Pyr), a Mannich base synthesized in China in the 1970s (3, 16), is being developed as a novel antimalarial for multidrug-resistant malaria. It demonstrates potent *in vitro* activity against erythrocytic stages of *Plasmodium falciparum* (8, 24, 26, 36), retaining efficacy against CQR isolates (12, 17, 18). Clinical trials have shown excellent efficacy of monotherapy against multidrug-resistant falciparum malaria (14, 24, 25), with the early therapeutic response faster when combined with artesunate (20). Phase III studies with a coformulation of Pyramax (Shin Poong Pharmaceuticals) containing artesunate plus pyronaridine have recently been completed (34).

Less is known of the antimalarial properties of pyronaridine against *P. vivax*, although early clinical studies in China demonstrated a rapid therapeutic response (3). To investigate the activity of pyronaridine against CQR *P. vivax*, we applied a modified schizont maturation assay on fresh field isolates from Papua, Indonesia, where CQR *P. vivax* is highly prevalent.

MATERIALS AND METHODS

Field location and sample collection. *Plasmodium* sp. isolates were collected from patients with uncomplicated malaria presenting to the Rumah Sakit Mitra Masyarakat (RSMM) hospital between January 2006 and July 2007. RSMM is situated on the southern coast of Papua, Indonesia, in a forested lowland area where malaria transmission is unstable, with an estimated annual incidence of 802 per 1,000 person years (divided 57:43 between *P. falciparum* and *P. vivax* infections) (11). Drug-resistant strains of *P. vivax* and *P. falciparum* are endemic to the area, with the risk of treatment failure reaching 65% within 28 days after chloroquine monotherapy for vivax malaria and 48% after chloroquine plus sulfadoxine-pyrimethamine (SP) for falciparum malaria (22). Patients with symptomatic malaria presenting to an outpatient facility were recruited into the study if singly infected with *P. falciparum* or *P. vivax* with a parasitemia between 2,000 μl^{-1} and 80,000 μl^{-1}. These criteria reflect the technical difficulties of reliably quantifying parasite stages using microscopy at low parasitemias. Although they may raise potential attrition bias, the criteria include the majority of patients presenting with clinical malaria in this region (geometric mean parasitemia, 1,600 to 3,000 μl^{-1}) (9, 21). Patients treated with antimalarials in the previous 3 weeks were excluded from the study. Venous blood (5 ml) was collected by venipuncture, host white blood cells were removed using a CF11 column, and packed infected red blood cells (IRBC) were used for the *in vitro* drug susceptibility assay.

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TABLE 1. Characteristics of isolates for which an *in vitro* assay was accomplished

Baseline characteristic	Value	
	P. falciparum	*P. vivax*
Total no. assayed	90	99
Median (range) delay from venipuncture to start of culture (h)	1.7 (0.8–4.6)	1.7 (0.3–4.6)
Median (range) duration of assay (h)	31 (24–53)	29 (24–56)
Geometric mean (95% CI), parasitemia (no. of asexual parasites/μl)	14,197 (12,093–16,667)	8,364 (7,184–9,741)
Median initial % (range) of parasites at ring stage	100 (93–100)	49 (0–99)
Mean (95% CI) % schizonts at harvest	61.9 (58.1–65.6)	44.8 (41.2–47.6)

In vitro drug susceptibility assay. The antimalarial susceptibilities of *P. vivax* and *P. falciparum* isolates were measured using a protocol modified from the WHO microtest as described previously (27, 28). In this test, drug activity is presented as inhibition of parasite growth from ring stage to schizont but does not quantify any activity on merozoites or reinvasion. Two hundred microliters of a 2% hematocrit blood medium mixture (BMM), consisting of RPMI 1640 medium plus 10% AB^+ human serum (*P. falciparum*) or McCoy's 5A medium plus 20% AB^+ human serum (*P. vivax*), was added to each well of predosed drug plates. Each drug plate contained 11 serial concentrations (2-fold dilutions) of the antimalarials, with maximum concentrations of 87 nM for pyronaridine, 5,910 nM for chloroquine, 557 nM for amodiaquine, 93 nM for artesunate, 338 nM for mefloquine, and 769 nM for piperaquine. The parasites were cultured to mature them in a candle jar at 37.5°C for 21 to 46 h. Incubation was stopped and the plates were harvested when >40% of ring stage parasites had reached the mature schizont stage in the drug-free control.

Thick blood films made from each well were stained with 5% Giemsa solution for 30 min and examined microscopically. Differential counts of 200 asexual parasites in both the preincubation and test slides were classified into ring stage (ring-shaped trophozoites without pigment), mature trophozoites (a single chromatin dot and hemazoin pigment visible), and schizonts (two or more chromatin dots).

To determine the effect of the antimalarial, the number of schizonts (≥5 chromatin dots visible per 200 asexual-stage parasites, with the stricter definition for the count improving assay accuracy) was determined for each drug concentration and normalized to the control well. Free merozoites and gametocytes were not included in the count. The dose-response data were analyzed using nonlinear regression analysis (WinNonLin 4.1; Pharsight Corporation), and the 50% inhibitory concentration (IC_{50}) was derived using an inhibitory sigmoid E_{max} model. Only IC_{50} *in vitro* data from predicted curves where the E_{max} (maximum effect) and E_0 (minimum effect) were within 15% of 100 or 0, respectively, were used.

Data analysis. Analysis was performed using SPSS for Windows (v. 15; SPSS Inc., Chicago, IL). The Mann-Whitney U test or the Kruskal-Wallis method was used for nonparametric comparisons. For categorical variables, percentages and corresponding 95% confidence intervals (CI) were calculated using Wilson's method. Proportions were examined using χ^2 with Yates' correction or by Fisher's exact test. The level of statistical significance was taken as a P value of <0.05, with Bonferroni correction for multiple comparisons.

Previous studies have highlighted the importance of the initial stage of the parasite and the duration of the assay for derived IC_{50}s. Therefore, the results for *P. vivax* were presented in a *post hoc* selection of isolates with a majority of ring stage parasites at enrollment and an assay duration between 30 and 50 h (27).

Ethical approval. Ethical approval for this study was obtained from the ethics committees of the National Institute of Health Research and Development, Ministry of Health, Indonesia, and the Menzies School of Health Research, Darwin, Australia.

RESULTS

Between January 2006 and July 2007, the *in vitro* susceptibility of pyronaridine was assessed in 221 patients with single-species infections by either *P. vivax* ($n = 117$) or *P. falciparum* ($n = 104$). Susceptibility profiles for the same isolates were also tested against chloroquine, amodiaquine, artesunate, mefloquine, and piperaquine. The baseline characteristics of these isolates are presented in Table 1. Adequate growth for harvest was achieved in 87% (90/104) of *P. falciparum* isolates and 85% (99/117) of *P. vivax* isolates, with a mean schizont count at harvest of 53% (95% CI, 50 to 56).

Initial stage of the parasite and *in vitro* susceptibility. There was a significant difference between the synchronicities of *P. falciparum* and *P. vivax* isolates. Whereas the median proportion of ring stages in the *P. falciparum* isolates was 100% (range, 93 to 100%), this proportion fell to 49% (range, 0 to 99%) in *P. vivax* isolates ($P < 0.0001$), with only 50% (50/99) of the isolates successfully processed having a ring-to-trophozoite ratio (RT ratio) greater than 1. The RT ratio for *P. vivax* at the start of culture was correlated significantly with the IC_{50}s for chloroquine ($r_s = -0.460$; $P < 0.001$) and mefloquine ($r_s = -0.295$; $P = 0.015$), but this was not apparent for pyronaridine, piperaquine, amodiaquine, or artesunate.

To investigate the stage-specific drug susceptibility in *Plasmodium*, isolates with greater than 90% rings were set up in culture in the presence of drug directly and again after culture in the absence of drug to achieve 90% trophozoites. Isolates assayed at ring stage had significantly lower IC_{50}s of pyronaridine than the same isolates assayed at trophozoite stage for *P.*

TABLE 2. *In vitro* susceptibilities for paired isolates tested at ring (>90% before culture) and trophozoite (>90% after culture in the absence of drug) stages

Drug	*P. falciparum*				*P. vivax*			
	n	Median IC_{50} (range) (nM)		*P*	*n*	Median IC_{50} (range) (nM)		*P*
		Rings	Trophozoites			Rings	Trophozoites	
Pyronaridine	11	1.6 (0.6–8.9)	8.0 (1.2–21.7)	0.015	8	2.5 (1.4–15.6)	4.7 (1.4–18.7)	0.085
Chloroquine	8	34.6 (11.9–55.0)	60.6 (19.6–157.8)	0.125	8	49.2 (18.3–171.8)	2427 (384–4457)	0.04
Amodiaquine	8	8.1 (2.6–16.2)	12.6 (0.4–22.4)	0.805	8	16.5 (6.7–37.9)	25.2 (12.5–59.9)	0.18
Mefloquine	11	4.9 (1.6–14.8)	8.5 (2.4–34.1)	0.04	8	7.9 (1.3–24.6)	12.4 (1.9–25.0)	0.615
Piperaquine	10	11.8 (2.7–30.4)	77.2 (13.6–1195)	0.025	8	14.0 (8.4–42.0)	21.7 (10.5–62.0)	0.085

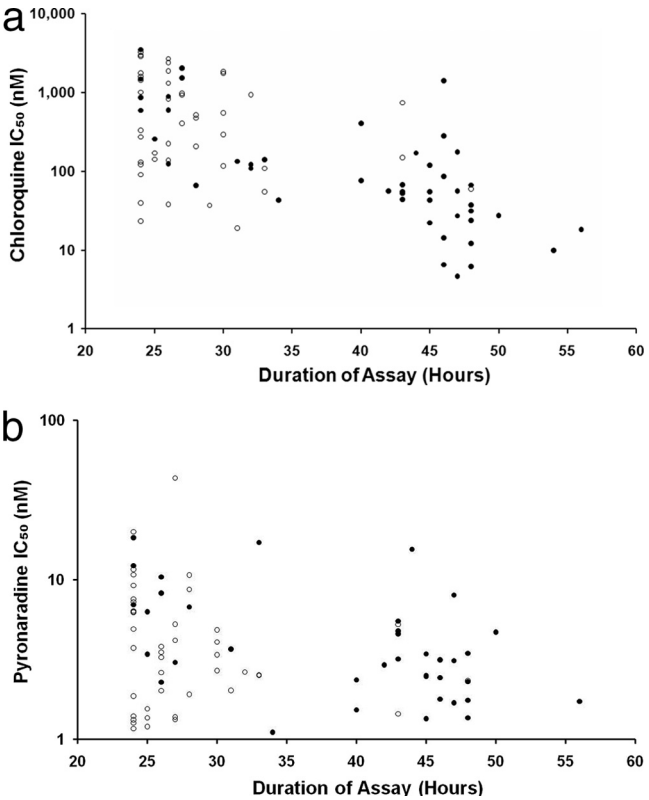

FIG. 1. Scatter plots of the duration of the assay with the derived *in vitro* susceptibility (IC_{50}) for chloroquine (a) and pyronaridine (b). Open circles, isolates initially predominantly at trophozoite stage; closed circles, isolates initially predominantly at ring stage.

falciparum (median, 1.6 nM [range, 0.6 to 8.9 nM] versus 8.0 nM [range, 1.2 to 21.7 nM]; $P = 0.015$), but not *P. vivax*. The derived IC_{50}s of *P. falciparum* were also significantly lower for mefloquine and piperaquine, whereas in *P. vivax*, differential drug activity was apparent only for chloroquine (Table 2).

Duration of the assay and *in vitro* susceptibility. The median time to reach the threshold for harvest was 31 h (range, 24 to 53 h) for *P. falciparum* and 29 h (range, 24 to 56 h) for *P. vivax* ($P = 0.505$). In *P. vivax*, the duration of the assay was highly correlated with the RT ratio prior to culture ($r_s = 0.645$; $P < 0.001$), but this was not apparent for *P. falciparum* isolates, which were predominantly at the ring stage prior to culture. A negative correlation between the duration of the assay and *P. vivax* IC_{50}s was observed for chloroquine ($r_s = -0.629$; $P < 0.001$) (Fig. 1a), pyronaridine ($r_s = -0.260$; $P = 0.05$) (Fig. 1b), amodiaquine ($r_s = -0.317$; $P = 0.01$), mefloquine ($r_s = -0.490$; $P < 0.001$), and piperaquine ($r_s = -0.432$; $P < 0.001$), but not artesunate ($r_s = 0.000$; $P < 0.995$). After the revised selection criteria were applied, 36 *P. vivax* isolates initially at majority ring stage (>50% rings) had assay durations between 30 and 50 h. In this reduced sample set, there was no significant correlation between the duration of the assay and the IC_{50} for any drug tested.

Antimalarial susceptibility. The overall median IC_{50}s are presented in Table 3. The IC_{50}s of pyronaridine for both, *P. falciparum* and *P. vivax* were significantly lower than those for all of the other drugs ($P < 0.001$), with the exception of artesunate, which had the lowest IC_{50} of any drug tested ($P < 0.001$). The pyronaridine susceptibility was positively correlated with chloroquine, amodiaquine, and piperaquine in both *P. falciparum* ($r_s = 0.449$ to 0.746; $P < 0.001$) and *P. vivax* ($r_s = 0.523$ to 0.721; $P < 0.001$) assays (Table 4). Whereas artesunate susceptibility in *P. falciparum* was correlated with pyronaridine ($r_s = 0.636$; $P < 0.001$) and mefloquine ($r_s = 0.286$; $P = 0.035$), this was not apparent in *P. vivax*.

DISCUSSION

In Southeast Asia and South America, *P. vivax* accounts for up to 50 to 70% of symptomatic malaria. Whereas the World Health Organization advocates the use of artemisinin combination therapies (ACTs) for *P. falciparum*, chloroquine remains the mainstay of treatment for *P. vivax*, with the inevitable consequence that in areas where both species are endemic a dual treatment policy is often necessary. Such an approach is being increasingly undermined by the emergence and spread of chloroquine-resistant *P. vivax*. Several countries where drug resistance is present in both species have chosen to implement a unified antimalarial policy (7). However, since the molecular mechanisms of drug resistance in *P. vivax* are clearly different from those in *P. falciparum* (15, 32), one cannot assume that the susceptibility of one species to a particular treatment regimen implies susceptibility in the other.

In vitro drug susceptibility testing is used routinely to monitor antimalarial drug resistance in *P. falciparum* and to screen for novel antimalarial compounds. Similar approaches in *P. vivax* are much more difficult, since unlike *P. falciparum*, this parasite preferentially invades young red blood cells, reducing

TABLE 3. Overall *in vitro* sensitivity for each drug according to the species tested

Drug	*P. falciparum*		*P. vivax*			
			All		>50% rings and assay duration 30–50 h	
	n	Median IC_{50} (range) (nM)	*n*	Median IC_{50} (range) (nM)	*n*	Median IC_{50} (range) (nM)
Pyronaridine	90	1.92 (0.24–13.8)	98	2.58 (0.13–43.6)	36	2.4 (0.13–17.2)
Chloroquine	90	43.6 (7.3–120.3)	90	141.5 (4.6–3506)	36	55.3 (4.6–1411)
Amodiaquine	89	5.7 (1.4–25.8)	97	14.0 (0.37–95.8)	37	12.0 (0.37–42.2)
Artesunate	71	0.68 (0.06–5.05)	91	1.03 (0.04–13.6)	30	1.27 (0.08–12.0)
Mefloquine	88	4.9 (0.32–28.8)	98	12.1 (0.81–175.6)	36	8.0 (0.81–29.8)
Piperaquine	89	17.1 (1.5–107.2)	97	24.8 (1.8–160.6)	36	19.6 (1.8–74.6)

TABLE 4. Correlation coefficients (r_s) for *in vitro* antimalarial susceptibilities in *P. falciparum* and *P. vivax*

Drugs	*P. falciparum*			*P. vivax*					
	All isolates			All isolates			≥50% rings and assay duration 30–50 h		
	Correlation	*P*	df	Correlation	*P*	df	Correlation	*P*	df
Pyronaridine, chloroquine	0.449	<0.001	89	0.339	0.005	88	0.510	0.01	33
Pyronaridine, amodiaquine	0.746	<0.001	88	0.721	<0.001	95	0.736	<0.001	34
Pyronaridine, artesunate	0.636	<0.001	70	0.564	<0.001	89	0.408	0.24	27
Pyronaridine, mefloquine	0.286	0.035	87	0.523	<0.001	97	0.332	0.22	34
Pyronaridine, piperaquine	0.621	<0.001	88	0.576	<0.001	96	0.611	<0.001	34

parasite growth and confounding continuous ex vivo culture (8, 35). To overcome this, short-term assays with field isolates of asexual parasites fresh from the human host have been used to evaluate the inhibitory effects of antimalarials on *P. vivax* (6, 28, 33). Our previous studies have demonstrated that isolates of *P. vivax* initially at the trophozoite stage are intrinsically resistant to chloroquine (27, 29), and indeed, the results of the current study confirm these findings. Since the synchronicity of infection varies between geographical locations and with the age of the patient, it is critical that the *in vitro* drug response be interpreted according to the initial stage of the parasite and the duration of the assay. The current *in vitro* susceptibility assay has shown utility in confirming the presence of emerging drug resistance (5, 10, 32), characterizing drug susceptibility profiles (5, 31), and screening for susceptibility to therapeutic agents (13). More recently, we have revised the assay criteria so that the quantification of parasite growth is restricted to parasites that have been exposed to the drug through all stages of the asexual life cycle (27). These more stringent criteria demand that the initial isolates be predominantly at the ring stage and be cultured for between 30 and 50 h. Under such conditions, any parasites initially at trophozoite stage will have matured to schizonts and ruptured and thus will not be quantified using microscopy-based quantification methods.

In the present study, we applied our modified schizont maturation test to assess the *in vitro* antimalarial activity of pyronaridine, an important new schizontocidal drug. Previous studies have documented the *in vitro* activity of pyronaridine in *P. falciparum*, highlighting high efficacies against both chloroquine-sensitive and -resistant strains (4, 12, 18, 26). Our study confirms similarly high activity against multidrug-resistant *P. falciparum* from southern Papua and also demonstrates its potency against highly drug-resistant strains of *P. vivax*, with IC_{50}s in the low nanomolar range. Compared to the other drugs tested, only artesunate showed greater antiparasitic activity.

In keeping with our previous studies, we found that antimalarial *in vitro* activity varied with the initial stage of the parasite, with trophozoites reaching the threshold for harvest more quickly and having higher derived IC_{50}s. This was particularly apparent for chloroquine in *P. vivax* but was also apparent, albeit to a lesser degree, in the activities of pyronaridine, piperaquine, and mefloquine. Analysis of *P. vivax* susceptibility was therefore restricted to the 37% of isolates initially predominantly at ring stage prior to culture, with assay durations of 30 to 50 h. There was significant correlation between pyronaridine IC_{50}s and the other drugs. Although this could suggest cross-resistance, only 26% of variation in activity could be explained by variation in chloroquine activity, with pyronaridine retaining extremely high activity against all isolates; the derived IC_{50} never exceeded 17.2 nM. The correlation coefficients were significantly higher between pyronaridine and amodiaquine (another quinoline-type Mannich base) and piperaquine (a bis-4-amino-quinoline), with variation in the activities of these compounds explaining 54% and 37% variation of pyronaridine, respectively.

Laboratory-adapted strains of *P. vivax* that can be used for screening novel antimalarial agents against *P. vivax* have yet to be developed. Our modified schizont maturation assay, carried out under field conditions in southern Papua, suggests that pyronaridine retains excellent susceptibility against multidrug-resistant strains of both *P. falciparum* and *P. vivax.* These results are reassuring as the novel artesunate-pyronaridine combination continues to be tracked in different endemic settings.

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