In vitro activity of pyronaridine against multidrug resistant
Plasmodium falciparum and P. vivax

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Financial Support: The Wellcome Trust (UK) and NHMRC (Australia).
Abstract

Pyronaridine, a Mannich base antimalarial, has demonstrated high in vivo and in vitro efficacy against chloroquine-resistant *Plasmodium falciparum*. Although this drug has potential to become a prominent artemisinin combination therapy, little is known about its efficacy against drug resistant *P. 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 IC$_{50}$ of pyronaridine was 1.92 nM [Range 0.24-13.8] against *P. falciparum* and 2.58 nM [Range 0.13-43.6] against *P. vivax*, with in vitro susceptibility correlating significantly with that for chloroquine, amodiaquine and piperaquine ($r_s$ = 0.45-0.62; $p<0.001$). *Plasmodium falciparum* parasites initially at trophozoite stage had higher IC$_{50}$s to pyronaridine compared to those exposed at the ring stage (8.9 nM [range 0.6-8.9] vs 1.6 [Range 0.6-8.9] respectively; $p=0.015$) although this did not reach significance for *P. vivax* (4.7 nM [range 1.4-18.7] vs 2.5 [Range 1.4-15.6] respectively; $p=0.085$). The excellent in vitro efficacy of pyronaridine against both chloroquine-resistant *P. vivax* and *P. falciparum* highlights the suitability of this drug as a novel partner for artemisinin-based combination therapy in regions coendemic for both species.

**Key Words**: malaria, *vivax, falciparum*, pyronaridine, resistance, Indonesia, in vitro
**Introduction**

Almost 40% of the world’s population is at risk of *P. vivax*, with an estimated 132 - 391 million clinical infections each year (19). Although chloroquine (CQ) remains the treatment of choice in most of the 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 South America (1). In Papua, Indonesia, the level of CQ resistance in *P. vivax* has reached levels precluding its use in most of the province (22, 30). There is an urgency to assess the efficacy of alternative antimalarial agents against drug resistant *P. vivax* and 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 *P. 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 co-formulation 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 spp. 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 malaria of 802 per 1000 person years (divided 57:43 between P. falciparum and P. vivax infections)(11). The area is endemic for drug resistant strains of P. vivax and P. falciparum, 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 of between 2,000µl^1 and 80,000µl^1. These criteria reflect the technical difficulties of reliably quantifying parasite stages using microscopy at low parasitaemias. Although they may raise potential attrition bias, the criteria will include the majority of patients presenting with clinical malaria in this region (geometric mean parasitaemia 1600 – 3000 ul^1)(9, 21). Patients treated with antimalarials in the past three weeks were excluded from this study. Venous blood (5 ml) was collected by venipuncture, host white blood cells removed using a CF11 column, and packed infected red blood cells (IRBC) were used for the \textit{in vitro} drug susceptibility assay.

\textit{In vitro} drug susceptibility assay

The antimalarial susceptibility of P. vivax and P. falciparum isolates was 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 µL of a 2% haematocrit Blood Media 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 pre-dosed 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. Parasites were cultured to mature the parasites in a candle jar at 37.5°C for 21 - 46 hours. Incubation was stopped and the plates 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 minutes and examined microscopically. Differential counts of 200 asexual parasites in both the pre-incubation and test slides were classified into ring stage (ring-shaped trophozoites without pigment), mature trophozoites (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, 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 IC50 value derived using an inhibitory sigmoid Emax model. IC50 in vitro data were only used from predicted curves where the Emax and E0 were within 15% of 100 or 0, respectively.
Data analysis

Analysis was performed using SPSS for Windows (vs 15, SPSS Inc, Chicago, Illinois, USA). The Mann-Whitney U test or Kruskal-Wallis method were used for nonparametric comparisons. For categorical variables percentages and corresponding 95% confidence intervals (95% CI) were calculated using Wilson’s method. Proportions were examined using \( \chi^2 \) with Yates' correction or by Fisher's exact test. The level of statistical significance was taken as \( p<0.05 \), with Bonferroni correction for multiple comparisons.

Previous studies have highlighted the importance of the initial stage of the parasite and duration of the assay on derived IC\(_{50}\)s. Therefore, the results of \( P. \) vivax were presented in a post hoc selection of isolates with majority of ring stage parasites at enrolment and assay duration of between 30 and 50 hours (27).

Ethical approval

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

Results

Between January 2006 and July 2007, the in vitro susceptibility of pyronaridine was assessed from 221 patients infected with either single-species infections of \( P. \) vivax (n=117) or \( P. \) falciparum (n=104). Susceptibility profiles on the same isolates were also tested against chloroquine, amodiaquine, artesunate, mefloquine and piperaquine. 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: 50-56].
**Initial Stage of parasite and in vitro susceptibility.** There was significant difference in synchronicity of *P. falciparum* and *P. vivax* isolates. Whereas the median proportion of ring stages in the *P. falciparum* isolates was 100% [range: 93-100], this proportion fell to 49% [range: 0-99] in *P. vivax* isolates (*p*<0.0001), with only 50% (50/99) of isolates successfully processed having a ring to trophozoites ratio (RT ratio) greater than 1. The RT ratio for *P. vivax* at the start of culture was correlated significantly with the IC₅₀ value for chloroquine (*r*ₚ = -0.460, *p*<0.001) and mefloquine (*r*ₚ = -0.295, *p*=0.015), but this was not apparent for pyronaridine, piperaquine, amodiaquine or artemunate.

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 IC₅₀s to pyronaridine compared to the same isolates assayed at trophozoite stage for *P. falciparum* (median 1.6nM [range: 0.6-8.9] vs 8.0nM [1.2-21.7]; *p*=0.015), but not *P. vivax*.

The derived IC₅₀s of *P. falciparum* were also significantly lower for mefloquine and piperaquine, whereas in *P. vivax* differential drug activity was only apparent for chloroquine (Table 2).

**Duration of assay and in vitro susceptibility.** The median time to reach the threshold for harvest was 31 hours [Range 24-53] for *P. falciparum* and 29 hours [range: 24-56] for *P. vivax*; *p*=0.505. In *P. vivax* the duration of assay was highly correlated with the RT ratio prior to culture (*r*ₚ=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 assay and *P. vivax* IC₅₀s was observed for chloroquine (*r*ₚ=−0.629, *p*<0.001, figure 1a), pyronaridine (*r*ₚ=−0.260, *p*=0.05, figure 1b), amodiaquine (*r*ₚ=−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 applying the revised selection criteria, 36 $P. vivax$ isolates initially at majority ring stage (Rings $>50\%$), had an assay duration between 30 and 50 hours. In this reduced sample set there was no significant correlation between the duration of assay and IC$_{50}$s for any drug tested.

**Antimalarial susceptibility.** The overall median IC$_{50}$ values 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 $P. falciparum$ ($r_s=0.449-0.746; p<0.001$) and $P. vivax$ ($r_s=0.523-0.721; p<0.001$) assays; see 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 SE Asia and South America, $P. vivax$ accounts for up to 50-70% of symptomatic malaria. Whereas the World Health Organisation 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 to that in *P. falciparum* (15, 32), one cannot assume that the susceptibility of one species to a particular treatment regimen infers susceptibility in the other.

*In vitro* drug susceptibility testing is used routinely to monitor antimalarial drug resistance in *P. falciparum* and 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 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 effect 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 is 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 the screening of 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 drug through all stages of the asexual life cycle (27). These more stringent criteria demand that the initial isolates are predominantly at the ring stage and cultured for between 30 and 50 hours. Under such conditions, any parasites initially at the trophozoite stage will have matured to schizonts, ruptured and thus not be quantified using microscopy-based quantification methods.

In the present study, we have 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 efficacy 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 artemunate 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 activity 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 duration 30-50 hours. There was a 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 exceeding 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 activity of these compounds explaining 54% and 37% variation of pyronaridine, respectively.

Laboratory adapted strains of *P. vivax* have yet to be developed that can be used for screening novel antimalarial agents against *P. vivax*. 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 artemunate-pyronaridine combination continues to be trailed in different endemic settings.
Acknowledgements
We are grateful to Lembaga Pengembangan Masyarakat Amungme Kamoro. The study was funded by the Wellcome Trust (ICRG GR071614MA and a Career Development Award to RNP), NHMRC (ICRG ID 283321 and Program and Fellowships to NMA and BR). We thank the Australian Red Cross Blood Service for supply of human sera for parasite culture.

Conflict of Interest
None Declared

Figure Legends

Figure 1.
Scatter plot of the duration of the assay with the derived in vitro susceptibility (IC_{50}) for chloroquine (1a) and pyronaridine (1b). Open circles = isolates initially predominantly at trophozoite stage; closed circles = isolates initially predominantly at ring stage.
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Table 1: Characteristics of isolates for which *in vitro* assay was accomplished

<table>
<thead>
<tr>
<th>Baseline Characteristic</th>
<th><em>P. falciparum</em></th>
<th><em>P. vivax</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Total assayed</td>
<td>90</td>
<td>99</td>
</tr>
<tr>
<td>Median [Range] delay from venipuncture to start of culture (hours)</td>
<td>1.7 [0.8-4.6]</td>
<td>1.7 [0.3-4.6]</td>
</tr>
<tr>
<td>Median [Range] duration of Assay (hours)</td>
<td>31 [Range 24-53]</td>
<td>29 [range: 24-56]</td>
</tr>
<tr>
<td>Geometric mean [95%CI], parasitemia (asexual parasites/µl)</td>
<td>14,197 [12,093-16,667]</td>
<td>8,364 [7,184-9,741]</td>
</tr>
<tr>
<td>Median initial % [Range] of parasites at ring stage</td>
<td>100 [93-100]</td>
<td>49 [0-99]</td>
</tr>
<tr>
<td>Mean [95% CI] schizont count at harvest</td>
<td>61.9% [58.1-65.6]</td>
<td>44.8% [41.2-47.6]</td>
</tr>
</tbody>
</table>

CI, Confidence Interval
Table 2: *In vitro* susceptibility for paired isolates tested at ring (>90% before culture) and trophozoite (>90% after culture in the absence of drug) stage

<table>
<thead>
<tr>
<th></th>
<th>P. falciparum</th>
<th></th>
<th>P. vivax</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rings</td>
<td>Trophozoites</td>
<td>Rings</td>
</tr>
<tr>
<td>Pyronaridine</td>
<td>11</td>
<td>1.6 [0.6-8.9]</td>
<td>8.0 [1.2-21.7]</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>8</td>
<td>34.6 [11.9-55.0]</td>
<td>60.6 [19.6-157.8]</td>
</tr>
<tr>
<td>Amodiaquine</td>
<td>8</td>
<td>8.1 [2.6-16.2]</td>
<td>12.6 [0.4-22.4]</td>
</tr>
<tr>
<td>Mefloquine</td>
<td>11</td>
<td>4.9 [1.6-14.8]</td>
<td>8.5 [2.4-34.1]</td>
</tr>
<tr>
<td>Piperaquine</td>
<td>10</td>
<td>11.8 [2.7-30.4]</td>
<td>77.2 [13.6-1195]</td>
</tr>
</tbody>
</table>
Table 3: Overall in vitro sensitivity for each drug according to species tested

<table>
<thead>
<tr>
<th></th>
<th>P. falciparum</th>
<th>P. vivax</th>
<th>P. vivax</th>
<th>P. vivax</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Median IC&lt;sub&gt;50&lt;/sub&gt; [Range]</td>
<td>N</td>
<td>Median IC&lt;sub&gt;50&lt;/sub&gt; [Range]</td>
</tr>
<tr>
<td>Pyronaridine</td>
<td>90</td>
<td>1.92 [0.24-13.8]</td>
<td>98</td>
<td>2.58 [0.13-43.6]</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>90</td>
<td>43.6 [7.3-120.3]</td>
<td>90</td>
<td>141.5 [4.6-3506]</td>
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<tr>
<td>Amodiaquine</td>
<td>89</td>
<td>5.7 [1.4-25.8]</td>
<td>97</td>
<td>14.0 [0.37-95.8]</td>
</tr>
<tr>
<td>Artesunate</td>
<td>71</td>
<td>0.68 [0.06-5.05]</td>
<td>91</td>
<td>1.03 [0.04-13.6]</td>
</tr>
<tr>
<td>Mefloquine</td>
<td>88</td>
<td>4.9 [0.32-28.8]</td>
<td>98</td>
<td>12.1 [0.81-175.6]</td>
</tr>
<tr>
<td>Piperaquine</td>
<td>89</td>
<td>17.1 [1.5-107.2]</td>
<td>97</td>
<td>24.8 [1.8-160.6]</td>
</tr>
</tbody>
</table>
Table 4: Correlation coefficients ($r_s$) for *in vitro* antimalarial susceptibilities in *P. falciparum* and *P. vivax*

<table>
<thead>
<tr>
<th></th>
<th><em>P. falciparum</em></th>
<th></th>
<th><em>P. vivax</em></th>
<th>Ringer ≥50% and Assay</th>
<th>Duration 30-50 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All Isolates</td>
<td>All Isolates</td>
<td></td>
<td>All Isolates</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Correlation</td>
<td>$p$</td>
<td>df</td>
<td>Correlation</td>
<td>$p$</td>
</tr>
<tr>
<td>Pyronaridine</td>
<td>0.449</td>
<td>&lt;0.001</td>
<td>89</td>
<td>0.339</td>
<td>0.005</td>
</tr>
<tr>
<td>Amodiaquine</td>
<td>0.746</td>
<td>&lt;0.001</td>
<td>88</td>
<td>0.721</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Artesunate</td>
<td>0.636</td>
<td>&lt;0.001</td>
<td>70</td>
<td>0.564</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mefloquine</td>
<td>0.286</td>
<td>0.035</td>
<td>87</td>
<td>0.523</td>
<td>&lt;0.001</td>
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<tr>
<td>Piperaquine</td>
<td>0.621</td>
<td>&lt;0.001</td>
<td>88</td>
<td>0.576</td>
<td>&lt;0.001</td>
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