**Plasmodium berghei ANKA: Selection of pyronaridine resistance in mouse model**


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Pyronaridine is a partner drug in, Pyramax®, a combination of artesunate (ASN)-pyronaridine (PRD) which was recently prequalified by WHO drug as a potential alternative for treatment of malaria in African setting. Pyronaridine is a mannich base, with a long half-life, thus predisposed to resistance. In this study, we selected pyronaridine resistance by submitting *Plasmodium berghei* ANKA line in vivo to increasing pyronaridine concentration for 20 successive passages over a period of six months. The effective doses that reduce parasitaemia by 50% (ED\(_{50}\)) and 90% (ED\(_{90}\)) determined in the standard four-day suppressive test for the parent line were 1.83 and 4.79 mg kg\(^{-1}\), respectively. After 20 drug pressure passages, the ED\(_{50}\) and ED\(_{90}\) increased by 66 and 40 fold, respectively. After dilution cloning, the parasites were grown in the absence of drug for five passages and cryo-preserving them at -80°C for at least one month, the resistance phenotypes remained stable. Thus, the resistant phenotype line could be used to explore genetic determinants associated with pyronaridine resistance; therefore, this strain represents a vital tool to study the mechanisms of resistance.

**Key words:** Malaria, pyronaridine, Pyramax®, resistance, *Plasmodium berghei* ANKA.

**INTRODUCTION**

Malaria is a global public health concern. The emergence of resistance, particularly in *Plasmodium falciparum*, has been a major contributor to the global resurgence of malaria in the last three decades (Marsh, 1998). In reality, *P.*
falciparum has developed resistance to nearly every anti-malarial drug introduced to date, compromising its control (White, 2004). Resistance arises via the selection of parasites bearing specific mutations, and is decisive in determining the effective life-time of anti-malarial agents. In response to resistance, artemisinin-based combination therapies (ACTs), that combines a semi-synthetic derivative of artemisinin, with a partner drug of a distinct chemical class, has been adopted for the treatment of falciparum malaria to delay the development of resistance (Nosten and White, 2007; Eastman, et al., 2005; WHO, 2003). However, there is a considerable concern that this will otherwise not happen. Previous studies on antimalarial resistance mechanisms have shown that drug elimination profile is one of the key factors in the emergence and selection of resistant phenotypes (Nzila et al., 2000; Watkins and Mosobo, 1993).

When drugs are used in combination, a discrepancy between their half-lives can have a substantial impact on the evolution of drug resistance. If one drug is rapidly eliminated, the other drug persists alone and new infections are exposed to sub-therapeutic level of drugs, a fact that promotes the development of resistance (Hastings, 2004). For instance, a combination of artesunate (ASN)-pyronaridine (PRD), commercialized as Pyramax®, was recently prequalified by WHO as a potential alternative for the treatment of malaria in Africa (Ramharter et al., 2008; Vivas et al., 2008). All artemisinin derivatives are characterized by a short half-life (WHO, 2006); while, pyronaridine is a long acting drug with half-life of 16 to 17 days (Sang and Pradeep, 2010). In this context, like any other antimalarial drug, the PRD is under intense selective pressure and resistance has, in the past, often developed rapidly.

Pyronaridine (Malaridine®) was first synthesized in China and introduced for the treatment of malaria as a single agent for over 30 years in certain malaria infested regions of China (Shao, 1990). Recently, interest has been renewed in pyronaridine as a possible partner for use in artemisinin-based combination therapy (ACT) for malaria treatment. Consequently, if strategy is to be devised to extend the expedient therapeutic lifetime of Pyramax®, there is a necessity to comprehend the molecular mechanisms of PRD resistance. However, to date, there is no well-established and characterized PRD-resistant P. falciparum strain, which could be used to study the mechanism of drug resistance.

In this study, we report the selection of pyronaridine resistance in vivo using piperquine-resistant P. berghei ANKA clone sensitive to pyronaridine. We then established the stability of the selected pyronaridine-resistant parasite. We selected stable pyronaridine-resistant phenotypes in six months. This study underscores the necessity to understand the genetic and molecular basis of PRD resistance, which would allow surveillance efforts for the emergence of parasites resistant to the partner drugs as a key component of the effective utilization of ACTs.

MATERIALS AND METHODS

Parasites and experimental animals

To select pyronaridine-resistant parasites, we used a transgenic ANKA strain of P. berghei expressing green fluorescent protein (GFP), resistant to piperquine but susceptible to pyronaridine, as described by Kibo et al. (2009). The cryopreserved parasites were first thawed and maintained by serial passage of blood from mouse to mouse at KEMRI animal house, Nairobi, Kenya. Before establishment of PRD resistance, we first established that the starting parasite (piperquine resistant clone) was sensitive to pyronaridine antimalarial drug by performing four suppressive day test and followed the development of the parasites for 15 days post infection.

Parasitized red blood cells (PRBCs) were collected from the donor mice with a rising parasitaemia of 5-10% and according to the level of parasitaemia, blood was diluted with phosphate saline glucose (PSG) buffer to reach approximately 2×107 PRBCs per 200 µl of the inoculum. The animals used were six to eight weeks old, randomized, male Swiss albino mice weighing (20±2 g), housed in the experimental room in a standard Macrolon type II cages clearly labeled with investigational details at 22°C and 60 to 70% relative humidity and fed on commercial rodent feedstuff and water ad libitum.

Preparation of test compound

Pyronaridine tetraphosphate synthesized by Shin Poong Pharm Co. (Seoul, Korea) was a gift from Professor Steve Ward, Liverpool School of Tropical Medicine, and Liverpool, UK courtesy of Dr. Alexis Nzila, KEMRI. Kenya. On the day of administration, pyronaridine tetraphosphate was freshly prepared by solubilizing it in solution consisting 70% Tween-80 (density=1.08gml⁻¹) and 30% ethanol (density=0.81 gml⁻¹) and diluted 10 fold with double distilled water.

Determination of 50 and 90% effective doses level (ED₅₀ and ED₉₀)

The effective doses that reduce parasitaemia by 50% (ED₅₀) and 90% (ED₉₀) were determined in the standard four day suppressive test (4-DT) as described by Peters (1975). Random-bred, male Swiss albino mice (20±2 g), five mice per dose group in four different doses and five mice in the control group were inoculated intraperitoneally each with 2×10⁷ PRBC in 200 µl inoculum on day zero (D₀). Drug was administered orally (p.o) at 4, 24, 48 and 72 h post infection. Thin blood films were prepared from tail snips on day 4 (D₄) post infection, fixed in methanol and stained for 10 min with freshly prepared in 10% (v/v) Giemsa solution. Parasitaemia was determined by microscopic examination of Giemsa-stained blood films taken on day 4 (96 h post infection). Microscopic counts of blood films from each mouse were processed using MICROSOFT® EXCEL (Microsoft Corp.), then percentage (%)) chemosuppression of each dose was determined as described elsewhere (Tona et al., 2001). 50 and 90% effective doses were estimated graphically using linear regression using version 5.5 of statistica 2000.

Procedures for exerting drug-selection pressure and assessing the level of resistance

In every passage (after inoculation with 2×10⁷ parasitized erythrocytes contained in 200 µl inoculum), three mice were infected with P. berghei and after attainment of >2% parasitaemia, the mice were treated orally with drug pressure dose of PRD. Throughout the selection of resistance, the drug concentrations were increased gradually.
depending on the growth of the parasites in the mice. During the first five passages, the drug pressure dose was increased by dose ranging from 5 to 10 mg/kg/day, while, in 5th to 20th passage, the PRD concentration was increased by 10 to 15 mg/kg/kg depending on the growth patterns of the parasites in mice. Acquisition of resistance was assessed after every five drug passages using standard 4-DT to confirm the response levels of the parasite to the pyronaridine compound. 4-DT permits the measurement of the ED\textsubscript{50} and ED\textsubscript{90}, as well as the index of resistance at the 50 and 90% levels (I\textsubscript{50} and I\textsubscript{90}), respectively. The indices of resistance (I\textsubscript{50} and I\textsubscript{90}) were defined as the ratio of the ED\textsubscript{50} or ED\textsubscript{90} of the resistant line to that of the parent strain (Merkli and Richle, 1980; Xiao et al., 2004).Resistance was classified into three categories based on earlier work (Merkli and Richle, 1980): (1) I\textsubscript{50} = 1.0, sensitive, (2) I\textsubscript{90} = 1.01-10.0, slight resistance, (3) I\textsubscript{50} = 10.01-100.0, moderate resistance and (4) I\textsubscript{90} > 100.0, high resistance.

**Stability tests**

The stability of PRD resistant line was evaluated by: (i) measuring drug responses after making five drug-free passages and (ii) freeze-thawing of parasites from -80°C stored for a period of four weeks followed by the measurement of effective doses in the 4-Day suppressive test. Stable resistance was defined as the maintenance of the resistance phenotype when drug-selection pressure was removed for at least five passages in mice (Gervais et al., 1999).

**Ethical consideration**

This study reported here was conducted in accordance with KEMRI guidelines, as well as, internationally accepted principles for laboratory animal use and care. Permission to carry out the study was granted by KEMRI’s Scientific Steering Committee and the Ethical Review Committee. (Study SSC No. 2457, 2013).

**RESULTS**

**Selection of resistance**

Effective doses, ED\textsubscript{50} and ED\textsubscript{90} of PRD against the parent parasite were 1.83 and 4.79 mg/kg/day, respectively. The parasite density was followed microscopically for 15 days post infection, the parasite were suppressed or cleared by 5 mg/kg/day of PRD (Figure 1A). We thus concluded that piperaquine-resistant clone is sensitive to PRD. As a result, this parasite clone was used as the parent strain to select PRD resistance. After 20 passages under PRD selective pressure, the ED\textsubscript{50} and ED\textsubscript{90} increased to 122.49 and 195.98 mg/kg/day, respectively, yielding I\textsubscript{50} of 66.93 and I\textsubscript{90} of 40.91. Such value of I\textsubscript{50,90} depicts that the starting parasites acquired resistance (Table 1) after several drug pressure passages.

Pyronaridine pressured lines were subjected to further five passages in untreated mice (five drug-free passages), after which they were tested for the drug responses. The selected line retained resistance levels yielding ED\textsubscript{50}=107.5 mgkg\textsuperscript{-1} and ED\textsubscript{90}=146.1 mgkg\textsuperscript{-1}. We then froze the parasite at -80°C, thawed after one month and inoculated into mice. The line retained the resistant-phenotype with marginal decrease in ED\textsubscript{50} and ED\textsubscript{90} of 73.48 and 107.50 mgkg\textsuperscript{-1}, respectively. Despite decrease in ED\textsubscript{50} and ED\textsubscript{90}, we concluded that the parasites retained resistance as I\textsubscript{90} was over 40 times compared to I\textsubscript{90} of parent strain. It is worthy to note that selection of resistance produced parasite populations with different susceptibility levels to the drug (Jiang et al., 2008) signifying different 90% index
Table 1. Changing response of the \textit{P. berghei} GFP ANKA resistant clone to PRD during exposure of the parasites to continuous drug pressure in mice.

<table>
<thead>
<tr>
<th>Passage no.</th>
<th>ED\textsubscript{50} (mg/kg)</th>
<th>I\textsubscript{50}</th>
<th>ED\textsubscript{90} (mg/kg)</th>
<th>I\textsubscript{90}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent parasite</td>
<td>1.83</td>
<td>1</td>
<td>4.79</td>
<td>1</td>
</tr>
<tr>
<td>5\textsuperscript{th}</td>
<td>7.12</td>
<td>3.90</td>
<td>11.38</td>
<td>2.38</td>
</tr>
<tr>
<td>10\textsuperscript{th}</td>
<td>25.82</td>
<td>14.11</td>
<td>69.43</td>
<td>14.49</td>
</tr>
<tr>
<td>15\textsuperscript{th}</td>
<td>92.05</td>
<td>50.3</td>
<td>168.98</td>
<td>35.27</td>
</tr>
<tr>
<td>20\textsuperscript{th}</td>
<td>122.49</td>
<td>66.93</td>
<td>195.98</td>
<td>40.91</td>
</tr>
<tr>
<td>20\textsuperscript{th} after dilution cloning</td>
<td>145.51</td>
<td>79.51</td>
<td>193.1</td>
<td>40.31</td>
</tr>
<tr>
<td>Drug-free passages (20\textsuperscript{th} passage)</td>
<td>107.5</td>
<td>58.74</td>
<td>146.1</td>
<td>30.5</td>
</tr>
<tr>
<td>20\textsuperscript{th} passages (after one month cryopreservation)</td>
<td>73.48</td>
<td>40.15</td>
<td>107.10</td>
<td>22.36</td>
</tr>
</tbody>
</table>

Figure 1B. Development of parasitaemia in the treated and untreated mice at different levels during the selection of PRD resistant \textit{Plasmodium berghei} GFP in mice. Parasitaemias were assessed after four days post-infection (in both control and treated groups) and mice were treated using a 4-day test (4-DT).

We have selected a stable pyronaridine resistant parasites using ST technique in 20 drug pressure passages over a period of six months. In this study, we used \textit{P. berghei} to select pyronaridine resistance as preliminary step towards
study of the molecular basis underlying PRD resistance. One early study found that resistance in *P. berghei* (ANKA) developed slowly to pyronaridine administered at 4 mg/kg, with no detectable high resistance within several passages (Shao and Xe, 1986). Peters and Robinson (1992) were able to derive pyronaridine-resistant *P. berghei* and *P. yoelii* strains by in vivo serial passage, applying drug at 3 or 10 mg/kg. However, resistance development was slow and was more difficult to achieve with the higher dose (Peters and Robinson, 1992). Interestingly, in our study we attained maximum level of resistance (>40 times the ED$_{90}$ of parent strain) by applying higher doses ranging from 5 to 100 mg/kg for over a period of 180 days. In fact, our study shows that it is more effective to select PRD resistance by maintaining high continuous drug pressure. This may be as a result of a genetic potentiation of the parasites to generate mutations in response to drug treatment (called the accelerated resistance to multiple drugs phenotype (Rathod et al., 1997) which might have occurred during generation of pyronaridine-resistant line. After making 5-drug free passages and upon revival of the parasites after one month of cryopreservation at -80°C, as shown in our data, there was a marginal degree in resistance. Observation from previous PRD stability studies on *P. berghei* (RP) and *P. berghei* (ANKA) have shown that the sensitivity started to return after making a number drug-free passages, after which the resistance remained stable (Xiao et al., 2004; Peters and Robinson, 1999).

Our study shows that PRD resistance may be selected within six months with a starting parasite line which is resistant to PQ. This study suggests that selective pressure for resistance to antimalarial combinations is exerted by the longer acting antimalarials, which persist in the body below effective concentrations long after treatment, promoting the selection of tolerance and ultimately resistance. Furthermore, studies suggest that even when true clinical resistance is not apparent, drug tolerance might be associated with specific biological processes in the parasite (Price et al., 2006; Nosten and White, 2007; Sisowath et al., 2007). If the concept of the existence of selective pressure because of long elimination half-life applies to all anti malarial drugs, it is expected that selective pressure to the ACTs, for instance, ASN-PRD would be exerted by PRD which is the partner drug with longer half-life (compared to the short acting artemisinin components; Artesunate). In the laboratories, two methods have been used to select resistant murine malaria parasites: the 2% relapse technique (2%RT) in which a single and high drug dose is administered at the time of each passage (Li et al., 1985) and the serial technique (ST), in which drug dose is gradually increased after each passage (Li, 1985; Li et al., 1985). Overall, the ST approach has proven to be more efficient to select for stable resistant strains than 2% RT (Peters and Robinson, 1999; Peters, 1999; Afonso et al., 2006). Using the ST technique, we have successfully established stable PRD-resistant *P. berghei* strains over a spell of six months of drug pressure.

In our study, we used rodent malaria parasite *P. berghei* as a surrogate for *P. falciparum* to study pyronaridine resistance. However, the mechanism of resistance in *P. falciparum* and murine *Plasmodium* species may be different. For instance, the mechanisms of resistance to chloroquine are different in *P. falciparum* and in murine malaria, and there is still a debate whether those of

**Figure 1C.** Selected resistant parasites subjected to PRD in parallel with their sensitive parental clone which had never been exposed to PRD compared with untreated group of mice. As anticipated, sensitive parental clone (Previously shown to be resistant to Piperaquine in Figure 1A) was cleared by 5 mg/kg while PRDR parasites tolerated 25 mg/kg (five times the drug concentration used to treat parent strain parasites), an indication that the parasites had acquired resistance.

![Graph showing percentage parasitaemia over time](image_url)
artemisinin derivatives will be similar (Cravo et al., 2003; Puri and Chandra, 2006). However, for drugs such as mefloquine, antifolates and atovaquone, similar mechanisms of resistance have been reported (Afonso et al., 2006; Carlton et al., 2001; Hunt et al., 2004a, b, 2007). Thus, this motivated the use of murine malaria in this study.

Structurally related compounds to pyronaridine, such as amodiaquine, demonstrated slight resistance against PQ-resistant line (Kibo et al., 2009), additionally, the AQ resistant line shown resistance to pyronaridine (Kibo et al., unpublished data). These studies suggest that the PRD resistance may share similar mechanism of resistance.

Conclusion

Selection of Pyronaridine resistance in Plasmodium berghei GFP ANKA strain using serial technique, suggests that PRD resistance develops rapidly as long as the selection pressure is maintained. From these results, we concluded that stable pyronaridine resistant P. berghei lines were selected. This stable pyronaridine resistance line could be used to facilitate molecular surveillance/monitoring and aid the development of strategies for the reversal of pyronaridine resistance.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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