Abstract
Chemotherapy remains central in the control of malaria. However, the rapid emergence and spread of antimalarial drugs remains a public health problem. To date, resistance to almost all available antimalarials has been reported. To counter this problem the resistance markers to existing drugs need to be fully understood. Pyramax®, a combination of artemisunate (ASN)-pyronaridine (PRD) was recently prequalified by WHO drug as a potential alternative for treatment of malaria in Africa. Artesunate is however partnered with a drug against which resistance may arise relatively quickly. We thus used rodent malaria parasite *Plasmodium berghei* as a surrogate for *P. falciparum* to study pyronaridine resistance. We selected resistance by submitting *P. berghei* ANKA in vivo to increasing pyronaridine concentration for twenty successive passages over a period of 6 months. The effective doses that reduces parasitaemia by 50% (ED_{50}) and 90% (ED_{90}) determined in the standard 4-Day Suppressive Test for the parent line were 1.83mgkg^{-1} and 4.79mgkg^{-1}. After twenty drug pressure passages the ED_{50} and ED_{90} increased by 66 and 40 folds respectively. We then assessed the stability of the resistant phenotypes by i) dilution cloning (ED_{50}=145.5mgkg^{-1}, ED_{90}=193.1mgkg^{-1}) ii) after growing them in absence of drug for five passages (ED_{50}=107.5mgkg^{-1}, ED_{90}=146.1mgkg^{-1}) and iii) after freezing the parasite at -80 degree for at least 1 month (ED_{50}=73.48, ED_{90}=107.10mgkg^{-1}). We concluded that stable pyronaridine resistant *P. berghei* lines were selected and could be used for elucidation of markers associated with pyronaridine resistance. The stability of the resistant phenotypes indicates that resistance mechanisms may be encoded in the cell genome.

Keywords: Malaria, pyronaridine, resistance, *Plasmodium berghei* ANKA

1.0 Introduction
Chemotherapy remains central in the control of malaria. However, the emergence of antimalarial resistance has rendered most antimalarial drugs ineffective. In fact, *Plasmodium falciparum* has developed resistance to nearly every anti-malarial drug introduced to date, compromising its control. 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 therapy (ACT), have been adopted for the treatment of *falciparum* malaria (Nosten and White, 2007; Eastman, et al., 2005; WHO, 2003), although this strategy is designed to reduce the chance of resistance emerging, there is considerable concern that this will otherwise not happen. Previous studies on antimalarial resistance mechanisms have shown that drug elimination profile is key in emergence and selection of resistant phenotypes (Nzila et al., 2000, Watkins, and Mosobo, 1993). For instance, a combination of artesunate (ASN)-pyronaridine (PRD), known as Pyramax®, was recently prequalified by WHO drug as a potential alternative for treatment of malaria in Africa (Ramharter et al., 2008). Artesunate is a short acting artemisinin derivative with half-life of less than 2hrs (WHO, 2006), while PRD is a long acting with half-life of 16 to 17days (Sang and Pradeep, 2010). Under these circumstances, the selective pressure for resistance would be primarily exerted by the PRD, leading to a rapid selection of PRD resistance. Therefore, the present study used rodent malaria parasite *P. berghei* ANKA to first select pyronadine resistance in vivo using piperaquine resistant *P. berghei* ANKA clone but sensitive to pyronaridine, and established the stability of the pyronaridine pressured parasite. We selected stable pyronaridine resistant phenotypes was selected in 6 months. These phenotypes form the basis for elucidation of mechanisms of resistance associated with PRD resistance.

2.0 Material and Methods
2.1 Parasites, Experimental Animals and Test Compounds
In this study piperaquine resistant parasites selected in Kiboi et al, 2009 were used. The parasites were first revived and maintained by serial passage of blood from mouse to mouse at KEMRI animal house. We first established that
the starting parasite were sensitive to piperaquine by performing 4 suppressive day test and development of the parasites followed for 15 days post infection.

On the day of administration, pyronaridine phosphate was freshly prepared by solubilizing it in solution consisting 70% Tween-80 (d=1.08g/ml-1) and 30% ethanol (d=0.81g/ml-1) and diluted 10 fold with double distilled water. Parasitized red blood cells (PRBCs) were collected from donor mice with a rising parasitaemia of 5-10% and according to the level of parasitaemia blood diluted with phosphate saline glucose (PSG) buffer to reach approximately 2×10^7 PRBCs per 200µl of the inoculum.

2.2 Determination of 50% and 90% Effective Doses Level (ED_{50} and ED_{90}).

The effective doses that reduces parasitaemia by 50% (ED_{50}) and 90% (ED_{90}) was determined in the standard 4-Day Suppressive Test (4-DT) as described by Fidock et al., 2004 Male Swiss albino mice, 5 mice per dose group- in four different doses and 5 mice in the control group was inoculated intraperitoneally each with 2×10^7 PRBC in 200µl inoculum on day zero (D_0). Drug were administered orally (p.o) at 4 hrs, 24 hrs, 48 hrs and 72 hrs post infection. Thin blood films was prepared from tail snips on day four (D_4) post infection, fixed in methanol and stained for 10 minutes with freshly prepared 10% (v/v) Giemsa solution. Parasitaemia was determined by microscopic examination of Giemsa-stained blood films taken on day 4. 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 by (Tona et al., 2001). 50% and 90% effective doses were estimated graphically using linear regression using version 5.5 of statistical 2000.

2.3 Procedures for Exerting Drug-Selection Pressure and Assessing the Level of Resistance

At every passage (After inoculation with 2x10^7 parasitized red blood cells contained in 200µl inoculum), 3 mice were infected with P. berghei and after attainment of >2% parasitaemia, the mice were treated orally with drug pressure dose of PRD. The dosages were increased gradually depending on the growth of the parasites. From the 1st passage and subsequent passages, the drug pressure dose was increased by dose ranging from 5-10mg/kg. day depending on the growth of the parasites. After every 5 passages of drug pressure, the level of resistance was assessed using 4-Day suppressive test.

2.4 Stability Tests

The stability of PRD resistant line was evaluated after measuring drug responses after making 5 drug-free passages, and freeze-thawing of parasites from -80 degree stored for a period of four weeks followed by measurement of effective doses in the 4DT. Stable resistance was defined as the maintenance of the resistance phenotype when drug-selection pressure was removed for at least 5 passages in mice (Gervais et al., 1999). Stable resistance was defined as the maintenance of the resistance phenotype when drug-selection pressure was removed for at least 5 passages in mice (Gervais et al., 1999).

2.5 Ethical Consideration

All animal work was conducted according to relevant national and international guidelines. The study received ethical approval from the Kenya Medical Research Institute National Ethical Review board (SCC 2457).

3.0 Results

3.1 Selection of Resistance

Effective doses, ED_{50} and ED_{90} of PRD against the parent parasite were 1.83 mg/kg. day and 4.79 mg/kg. day, respectively . The parasite density was followed microscopically for 15 days post infection, the parasite were suppressed/cleared by 5mg/kg. day of PRD (Figure 1). It was concluded that PQ (mpr) clone is sensitive to PRD, and hence used this parasite clone to select PRD resistance. After 20 passages under PRD selective pressure, the ED_{50} and ED_{90} increased to 122.49 and 195.98 mg/kg. day, respectively, yielding I_{50} of 66.93 and I_{90} of 40.91. Such value of I_{50/90} depicts that the starting parasites acquired resistance to the drug (Table 1).

The effective doses that reduce parasitaemia by 50% (ED_{50}) and 90% (ED_{90}) and as 50% (I_{50}) and 90% (I_{90}) indexes of resistance (I_{50} and I_{90}), defined as the ratio of the ED_{50} or ED_{90} of the resistant line to that of the parent strain).
Table 1: Summary of changing response of the *P. berghei* ANKA to PRD during exposure of the parasites to continuous drug pressure

<table>
<thead>
<tr>
<th>Passage No.</th>
<th>ED$_{50}$ (mg/kg)</th>
<th>I$_{50}$</th>
<th>ED$_{90}$ (mg/kg)</th>
<th>I$_{90}$</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$^{th}$</td>
<td>7.12</td>
<td>3.90</td>
<td>11.38</td>
<td>2.38</td>
</tr>
<tr>
<td>10$^{th}$</td>
<td>25.82</td>
<td>14.1</td>
<td>69.43</td>
<td>9</td>
</tr>
<tr>
<td>15$^{th}$</td>
<td>92.05</td>
<td>50.3</td>
<td>168.98</td>
<td>35.2</td>
</tr>
<tr>
<td>20$^{th}$</td>
<td>122.4</td>
<td>66.9</td>
<td>195.98</td>
<td>40.9</td>
</tr>
<tr>
<td>20$^{th}$ after Dilution cloning</td>
<td>145.5</td>
<td>79.5</td>
<td>193.1</td>
<td>40.3</td>
</tr>
<tr>
<td>Drug free passages (20$^{th}$ passage)</td>
<td>1</td>
<td>1</td>
<td>193.1</td>
<td>1</td>
</tr>
<tr>
<td>20$^{th}$ passages (after 1 month cryopreservation)</td>
<td>107.5</td>
<td>4</td>
<td>146.1</td>
<td>30.5</td>
</tr>
<tr>
<td></td>
<td>73.48</td>
<td>40.1</td>
<td>107.10</td>
<td>22.3</td>
</tr>
</tbody>
</table>

Pyronaridine pressured lines were subjected to a further 5 passages in untreated mice, after which they were tested for the drug responses. The selected retained resistance levels ED$_{50}$=107.5mgkg$^{-1}$ and ED$_{90}$=146.1mgkg$^{-1}$, we then froze the parasite at -80 degree, thawed after 1 month and inoculated into mice, again the lines retained the phenotype though with marginal decrease in ED$_{50}$ and ED$_{90}$ of 73.48 mgkg$^{-1}$ and 107.50 mgkg$^{-1}$, respectively. Selection of resistance produces parasites population with different susceptibility levels to the drug (Jiang et al., 2008) signifying different 90% index of resistance (I90), one with higher value (minority population) and another with lower value (dominant population) (Nzila and Mwai, 2009). We therefore dilution cloned the pyronaridine resistant lines and then determined drug profiles, the clone retained resistance yielding ED$_{50}$=145.5mgkg$^{-1}$, ED$_{90}$=193.1mgkg$^{-1}$. ED$_{90}$=146.1mgkg$^{-1}$.

![Figure 1: Parasitaemia development in the treated and untreated mice in a group of four mice taken on day 4,7,11 and 15 post infection before the start of the pyronaridine drug selection pressure](image-url)
Establishment of resistance throughout the selection pressure

The resistance indices $I_{50}$ and $I_{90}$ is defined as the ratio of the ED$_{50}$ or ED$_{90}$ of the resistant line to that of the parent strain in development of resistance to PRD after every five passage of over the selection pressure period of 6 months assessed by 4-suppressive day test.

Selection of resistance produces parasites population with different susceptibility levels to the drug signifying different 90% index of resistance ($I_{90}$), one with higher value (minority population) and another with lower value (dominant population) (Nzila and Mwai, 2010). To determine the genetic organization of the resistant parasites genotype, the resistant lines need to first (i) be clone diluted to generate a genetically homogenous parasite population (Rosario, 1981) and (ii) assess drug response profiles of the clone demonstrating comparable growth pattern as the sensitive progenitor.

![Figure 2: Establishment of resistance throughout the selection pressure](image)

Figure 3: Shows results when parasites were subjected to PRD in parallel with their sensitive parental clone which had never been exposed to PRD
As anticipated, starting parasite and sensitive strain were cleared by 25mg/kg day, indeed, no parasites were detected in mice infected with sensitive parasite over the 15 day p.i. follow up period and PRD clone grown in presence of 50mg/kg day.

4.0 Discussion

We have selected a stable pyronaridine resistant parasites using ST technique in 20 drug pressure passages over a period of 6 months. In this study, we used 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). However, higher doses were more effective at selecting for resistance (Xiao et al., 2004, Shao and Xe, 1986), conforming with our studies where we have selected PRD resistance by increasing dose concentration gradually from lowest dose (5mg/kg.day) to highest dose 100mg/kg.day which was maintained from 15th passage to 20th. However, higher doses were more effective at selecting for resistance (Shao and Xe, 1986), thus, conforming to our study where we maintained high continuous drug pressure of PRD to select resistance. In our stability studies after making 5-drug free passages and upon revival of the parasites after 1 month of cryopreservation at -80 degree, as shown in our data, there was marginal degree in resistance. Observation from previous PRD stability studies P. berghei (RP) and of P. berghei (ANKA), 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 6 months with a starting parasite line which is resistant to PQ. This study suggest that selective pressure for resistance to antimalarial combinations is exerted by the longer acting antimalarials, which persists in the body below effective concentrations long after treatment, promoting the selection of tolerance and ultimately resistance. Further, studies suggest that 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 antimalarials, it is expected that selective pressure to the ACTs, for instance, ASN-PRD would be exerted by PRD which is the partner drugs with longer half live (compared to the short acting artemisinin components; Artesunate).

In 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 6 months of drug pressure.

Although in this study we used rodent malaria parasite Plasmodium berghei as a surrogate for P. falciparum to study pyronaridine, 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 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; Cravo et al., 2003; Hunt et al.,2007, 2004a, b). Thus, this motivated the use of murine malaria in this study we hope this could provide valuable insights on the mechanisms of resistance to pyronaridine.
5.0 Conclusion
Selection of Pyronaridine resistance in *Plasmodium berghei* GFP ANKA strain using serial technique, suggest 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.

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