***African Journal of Pharmacology and Therapeutics*** Vol. 1 No. 2 Pages 35-40, 2012
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Research Article

# Lumefantrine-resistant and Piperaquine-resistant *Plasmodium berghei* show cross-resistance to Primaquine but not to Atovaquone

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**Background:** Malaria affects 300-500 million people annually and kills more than 1 million, with majority of the clinical cases and deaths occurring in Sub-Saharan Africa. Rapid development of drug resistance remains a major challenge in malaria control and has lead to use of combined antimalarial therapies. Resistance to an antimalarial drug may however, be selected for by another drug in which the mechanism of resistance is similar.

**Objective:** This study sought to establish cross-resistance patterns between four antimalarials namely atovaquone (ATQ), primaquine (PMQ), lumefantrine (LM) and piperaquine (PQ) using murine malaria models.

**Method**: The activities of ATQ and PMQ against drug sensitive, PQ and LM-resistant *Plasmodium berghei* lines was assessed using the 4-day test and 90% index of resistance ($I_{90}$) determined.

**Results:** Analysis of cross-resistance patterns showed a significant decrease in PMQ sensitivity ($I_{90}$ of 6.39), and a slight but not significant decrease in ATQ ($I_{90}$ of 1.19) activity towards the LM-resistant *P.berghei* ANKA.

**Conclusion**: PQ-resistance in *P. berghei* is associated with a significant resistance of PMQ ($I_{90}$ of 12.22) and a slight, though not significant reduction in ATQ ($I_{90}$ of 1.27) efficacy.

**Key words:** *Plasmodium berghei*, resistance, piperaquine, lumefantrine, primaquine, atovaquone

**Received**: February, 2012
**Published**: July, 2012

## 1. Introduction

Malaria is the most significant parasitic disease in many countries, with about 41% of the global population affected, mostly in sub-Saharan countries. In the last two decades, mortality and morbidity from the disease have increased, and this is attributed to the rise in resistance to existing antimalarial drugs. Resistance to all known antimalarial drugs, with the exception of the artemisinin derivatives, has developed to various degrees in several countries (Bloland, 2001). However, a few cases of decrease in artemisinin sensitivity have been reported in French Guyana and Senegal (Jambou et al, 2005). More recently, evidence of artemisinin-based combination therapy (ACT) resistance has been recorded in Cambodia (Lim et al, 2009; Noedl et al,

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2008; Rogers et al, 2009). Substantial progress has been made in understanding the molecular mechanisms underlying resistance of Plasmodium species to antimalarial drugs (Mehlotra et al, 2009). For instance, single and gene copy number mutations in *Plasmodium falciparum* chloroquine-resistance transporter (*Pfcrt*) and *P. falciparum* multidrug resistance transporter (*Pfmdr1*) genes have been identified in chloroquine (CQ) and mefloquine (MQ) resistance, respectively (Price et al, 2004). Other mutations, such as in the *P. falciparum* ATPase (*Pfatp6*) gene, (Valderramos and Fidock, 2006; Woodrow and Krishna, 2006), dihydrofolate reductase (*Pfdhfr*) gene, and dihydropteroate synthase (*Pfdhps*) gene (Hyde, 2007) are evolving as potential mechanisms of resistance to artemisinins, pyrimethamine and sulfadoxine, respectively.

As a way of fighting malaria, drug resistance studies focusing on the existing and new antimalarial drugs have been emphasized. Inducing and studying resistance to these drugs will lead to development of the most effective drug combinations that will prevent the occurrence of resistance. Furthermore, it will make it possible to test new molecules on resistant strains, identify new targets as well as predict resistance to new compounds (Witkowski et al, 2009).

Induction of resistance *in vitro* generally gives reliable results in comparison to field data. However, its data is not complete as they do not mimic the disease context, which is only possible with *in vivo* models. No *in vivo* rodent malaria model is ideal for studying resistance to all antimalarial compounds, thus the relevant pairing of Plasmodium species and drug molecule must be carefully chosen (Witkowski et al, 2009). The best approach would be to combine *in vitro* and *in vivo* models of *P. falciparum* in a pathophysiological context. Chimeric mice containing human bone marrow, and thus able to be infected with *P. falciparum* could be an exciting development to study and induce resistance (Moreno et al, 2007).

Therefore, to understand the mechanism of resistance to antimalarial drugs, drug resistant mutants have been induced *in vivo* using rodent malaria parasites. However, resistance to one antimalarial drug may be selected for by another drug in which the mechanism of resistance is similar, a phenomenon known as cross-resistance (White, 2004). Our previous study showed that selection of stable PQ-resistant and LM-resistant *P. berghei* ANKA lines is associated with significant decrease in CQ and AQ activity (Kiboi et al, 2009). CQ, AQ and PQ are aminoquinoline derivatives, thus they could be sharing mechanism(s) of resistance as well.

PMQ has no clinical utility as a blood schizonticide. However, the little activity it does possess against the erythrocytic form of the parasite may derive from an oxidative stress mechanism since it is well known that PMQ, largely via its hydroxylated metabolites, stimulates the hexose monophosphate shunt, increases hydrogen peroxide and methemoglobin production, and decreases glutathione levels in the erythrocyte (Vennerstrom et al, 1999). In mechanical infections involving rodent malaria parasites, like in the present study, there are no liver stage parasites, thus any PMQ activity could be attributed to oxidative stress mechanism.

ATQ is a hydroxynapthoquinone currently used for the treatment of opportunistic infections in immunosuppressed patients. It acts by collapsing the mitochondrial membrane potential, and therefore inhibiting parasite respiration (Srivastava et al, 1997). Significantly, PMQ and ATQ do not share mode of action with either LM or PQ. The focus of this study was to assess cross-resistance with drugs whose mode of actions differ significantly from that of PQ and LM.

This study assessed PMQ and ATQ on stable PQ-resistant and LM-resistant *P. berghei* ANKA lines (Kiboi et al, 2009) with a view to predicting cross resistance patterns as well as possible mechanism(s) of resistance.

## 2. Methods

### 2.1 Animals and parasites

Female Swiss albino mice weighing 18 - 22 g used in this study were obtained from a breeding colony kept at Kenya Medical Research Institute (KEMRI). In addition, PQ-resistant and LM-resistant *P. berghei* lines selected and cryopreserved at Centre for Traditional Medicine and Drug Research, KEMRI (Kiboi et al, 2009) as well as *P. berghei* ANKA–GFP (MRA-865, MR4, and ATCC® Manassas, Virginia) sensitive to LM and PQ, were used in the current study.

### 2.2 Preparation of drugs

ATQ and PMQ were gifts from Professor Steve Ward, Liverpool School of Tropical Medicine, and Liverpool, UK. Each drug was freshly prepared by solubilizing in solvent consisting of 70% Tween-80 and 30% ethanol and diluted tenfold with double distilled water.

### 2.3 Animal infection and treatment

Three groups of randomly selected female Swiss albino mice were infected intraperitoneally with $2 \times 10^7$ parasitized red blood cells (PRBCs) containing drug sensitive, LM-resistant, and PQ resistant *P. berghei* strains, respectively. Fifteen groups (five per parasite strain) was then treated orally with five different doses of PMQ (10, 5, 2.5, 1.25, and 0 mg/kg) at 4, 24, 48 and 72 hours post infection. Another fifteen groups of infected mice was similarly treated with five different doses of ATQ (0.125, 0.0625, 0.032, 0.016 and 0 mg/kg). All the groups used consisted of five mice each and care was taken to minimize animal stress.

### 2.4 Determination of $ED_{90}$ and $ED_{99}$

Parasitaemia in each group of mice was evaluated on day 4 (96 hours post infection) using Giemsa-stained thin blood smears made from tail blood. Mean parasitaemia was obtained for each dosage group of mice on day 4. Percentage chemosuppression for each drug was then calculated as described by Tona et al, 2001). The 90% effective level ($ED_{90}$) and $ED_{99}$ were calculated using linear regression (Xiao et al, 2004).

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### 2.5 Data and statistical analysis

Data was recorded in Microsoft® Office Excel® (2007) spreadsheet, analyzed using linear regression (Statistica 5.5, Statsoft Inc. 2000) so as to estimate $ED_{90}$ and $ED_{99}$ in mg/kg.day. Indices of resistance, $I_{90}$ and $I_{99}$, were calculated from the ratio $ED_{90}$ or $ED_{99}$ of resistant parasites to that of their drug sensitive parent line respectively (Merkli and Richle, 1980; Xiao et al, 2004). Statistical analyses were carried out using the Student t-test (Minitab Statistical Software, State College, PA, U.S.A.).

### 2.6 Ethical considerations

The study was conducted in accordance with KEMRI guidelines on animal care and use. Additionally; the study followed the internationally accepted principles for laboratory animal use and care, as found in Council for International Organizations of Medical Sciences (CIOMS) guidelines (CIOMS, 1986). Permission to carry out the study was granted by KEMRI'S Scientific Steering Committee and the Ethical Review Committee. (Study SSC No. 1501, 2009).

## 3. Results

Cross resistance was classified into three categories: $I_{90}$ ≤1.00 was considered as sensitive, $I_{90}$ of 1.01 - 5.00 as slight cross resistance, $I_{90}$ ≥ 5.01 as high (Li, 1985; Li et al, 1985).

The LM-resistant *P.berghei* ANKA luciferase parasite retained relative susceptibility to ATQ ($I_{90}$ 1.19) (p = 0.996) but showed a high but not significant cross-resistance towards PMQ ($I_{90}$ of 6.39) (p = 0.090) (**Table 1**).

Evolution and establishment of PQ resistance in *P. berghei* was associated with 12.2-fold loss of PMQ efficacy (p = 0.050) as well as a slight but not significant reduction in ATQ activity ($I_{90}$ 1.27) (p = 0.351) (**Table 2**).

**Table 1:** Summary response of LM-resistant *P. berghei* luciferase line to PMQ and ATQ

| Antimalarial drug | $ED_{90}$ (mg/kg.day for 4 days) | | Index of resistance ($I_{90}$) | *P*-value |
|---|---|---|---|---|
| | Sensitive strain | Resistant line | | |
| Primaquine | 1.33 | 8.49 | 6.39 | 0.090 |
| Atovaquone | 0.10 | 0.12 | 1.19 | 0.996 |

*Data are presented as effective doses that reduce parasitaemia by 90% ($ED_{90}$) and as 90% indexes of resistance ($I_{90}$) defined as the ratio of the $ED_{90}$ of the resistant line to that of the parent strain.*
*Differences between parent and resistant lines were analyzed by Student's t-test: p ≤ 0.05 interpreted as statistically significant*

**Table 2:** Summary response of PQ-resistant *Plasmodium berghei* ANKA line to PMQ and ATQ

| Antimalarial drug | $ED_{90}$ (mg/kg.day for 4 days) | | Index of resistance ($I_{90}$) | *P*-value |
|---|---|---|---|---|
| | Sensitive strain | Resistant line | | |
| Primaquine | 0.78 | 9.58 | 12.22 | 0.050 |
| Atovaquone | 0.10 | 0.13 | 1.27 | 0.351 |

*Data are presented as effective doses that reduce parasitaemia by 90% ($ED_{90}$) and as 90% indexes of resistance ($I_{90}$) defined as the ratio of the $ED_{90}$ of the resistant line to that of the parent strain.*
*Differences between parent and resistant lines were analyzed by Student's t-test: p ≤ 0.05 interpreted as statistically significant*

## 4. Discussion

The genetic events conferring antimalarial drug resistance are spontaneous, rare and independent of the drug used. Resistance to one drug may be selected for by another drug in which the mechanism of resistance is similar, a phenomenon known as cross-resistance (White, 2004).

LM is an arylaminoalcohol closely related to MQ, halofantrine (HF) and pyrimethamine (PYR) (Schlitzer, 2008). LM binds to toxic heme preventing the polymerization of heme to non-toxic waste haemozoin (Toovey and Jamieson, 2004). A strain of *P. berghei* showing resistance to MQ was obtained through the 2 % relapse time technique (Peters et al, 1977). This strain showed cross-resistances to quinine (QN), HF and artemisinin (ART), and manifested a 3.6-fold amplification of the *Pbmdr* gene (Gervais et al, 1999). The MDR1 channel was a major protagonist of this resistance pattern, a finding corroborated by other data concerning MQ-resistance in *P. falciparum* (Cowman et al, 1994; Nishiyama et al, 2004; Price et al, 1999). Cross-resistance exists between MQ and AQ in *P. berghei*, but has not yet been found in *P. falciparum* (Witkowski et al, 2009).

The copy number of *Pfmdr1* has been reported to increase with the use of LM in field isolates in Thailand (Price et al, 2006), and a decrease in copy number was found to heighten *in vitro* LM susceptibility in laboratory selected parasites (Duraisingh and Cowman

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2005; Sidhu et al, 2006). These observations indicate that *Pfmdr1* orthologue *Pbmdr1* will likely contribute to LM resistance in *P. berghei*.

PQ is a bis-chloroquine derivative (Davis et al, 2005a; Raynes, 1999). It inhibits heme digestion in the parasite food vacuole (WHO, 2003**;** Davis et al, 2005b**;** Ridley, 2002). No gene or gene candidate has been associated with PQ resistance in *P. falciparum*. Therefore, molecular analysis of PQ-resistant *P. berghei* line could give a useful insight into mechanism of *P. falciparum* resistance (Kiboi et al, 2009). However, it is instructive to note that resistance mechanism in *P. falciparum* and *P. berghei* may not always be the same (Afonso et al, 2006; Carlton et al, 2001; Hunt et al, 2004a; Hunt et al, 2004b; Hunt et al, 2007).

The current study provides possible indications as to the potential concerns that should be considered when antimalarials PMQ and ATQ are deployed in places where LM and PQ-resistant malaria has been demonstrated. This was done using selected LM and PQ-resistant *P. berghei* lines and thereafter associating or extrapolating the findings to the human Plasmodium malaria. PMQ displayed a decrease in response against LM-resistant *P. berghei* line. Previous studies showed that CQ-resistance in *P. berghei* is correlated with cross-resistance to PMQ (Peters, 1965). According to our previous study LM-resistance in *P. berghei* was linked to CQ-resistance (Kiboi et al, 2009). Findings from this study suggest the involvement of a similar resistance mechanism for LM and PMQ in *P. berghei*. Some studies, however, indicate that PMQ inhibits MDR1-related and multidrug resistance protein (MRP1)-related drug transport without being a substrate (Hayeshi et al, 2006; Wu et al, 2005).

Efficacy of ATQ against LM-resistant *P. berghei* was maintained. As a result, ATQ and LM may not be sharing a resistance mechanism. Unlike LM, ATQ acts by collapsing the mitochondrial membrane potential, thus inhibiting parasite respiration (Srivastava et al, 1997). ATQ resistance in *P. falciparum* is attributed to reduced affinity to its target in the parasite (Foote and Cowman, 1994; Ward et al, 1995). ATQ is effective against CQ-resistant *P. falciparum*, (Susan et al, 2005; Steffen et al, 2002). In our case ATQ manifested high efficacy against LM-resistant *P. berghei*, which was also associated with CQ-resistance (Kiboi et al, 2009). The findings lend credence to the fact that ATQ retains activity against strains of malaria parasites resistant to structurally and functionally diverse antimalarial drugs.

The current study established a significant decrease in activity of PMQ towards PQ-resistant line. CQ resistance in *P. berghei* is correlated with cross-resistance to PMQ (Peters, 1965). Our earlier findings showed significant cross-resistance of PQ resistant line and CQ (Kiboi et al, 2009), an indication that CQ may be sharing a resistance mechanism with PQ. And if indeed this is true, our present study seems to confirm it. However, there is evidence that PMQ acts as a CQ-resistance reversing agent for *P. falciparum* in culture (Egan, 2006), implying that in *P. falciparum* the mechanism of resistance of CQ and PMQ may be different.

The PQ-resistant *P. berghei* displayed a slight but insignificantly reduced sensitivity to ATQ. ATQ is effective against CQ-resistant *P. falciparum* (Susan et al, 2005; Steffen et al, 2002). Data from our previous study suggests that, in *P. berghei*, CQ could be sharing resistance mechanism with PQ (Kiboi et al, 2009). Therefore, the current findings seem to agree with the former results. Most importantly, our findings strengthen the idea that development of PQ-resistant *P. berghei* line involves a gene which causes CQ-resistance in both *P. berghei* and *P. falciparum*.

## 5.0 Conclusion

Analysis of cross-resistance pattern of the study showed that ATQ retained potency against both LM and PQ-resistant *P. berghei* lines. However, the study reports a significant decrease in PMQ activity towards the two resistant lines.

When our findings were analyzed and related to previous studies involving *P. falciparum* malaria, it can be deduced that the mechanisms of resistance in the selected PQ-resistant *P. berghei* strain and in *P. falciparum* could be significantly related. Furthermore, our findings support the use of mouse model as a surrogate in studying drug resistance for PQ and LM.

## Conflict of Interest declaration

The authors declare no conflict of interest

## Acknowledgements

This work was supported in part by the European and Developing Countries Clinical Trials Partnership (EDCTP) and Global Health Research Award GHRA-06-03 from the Health Research Board (Ireland) to Angus Bell and Alexis Nzila. Atovaquone and primaquine were gifts from Professor Steve Ward, Liverpool School of Tropical Medicine, and Liverpool, UK.

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