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Plasmodium berghei ANKA: Selection of resistance to piperaquine and lumefantrine in a mouse model.

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well established, the degree of $ED_{50}/_{90}$ decrease is small, the strains remained resistant to the drugs, as our data show.

Evaluation of cross-resistance patterns revealed that PQ and AQ retain potency against the LM resistant parasite line. LM is an arylaminoalcohol closely related to mefloquine (MQ), halofantrine and pyronaridine (Schlitzer 2008). PQ, AQ and CQ are 4-aminoquinoline derivatives, and are likely to share a similar mechanism of action (Raynes 1999). Resistance to CQ and AQ in *P. falciparum* is reported to be inversely correlated with resistance to arylaminoalcohols (Duraisingh and Cowman 2005), and the selection of the resistance to arylamino-alcohol MQ results in an increase in CQ sensitivity (Cowman et al., 1994; Peel et al., 1993). In our experiments, LM-resistance was not associated with a decrease in PQ efficacy. Similarly, the efficacy of the 4-aminoquinolines AQ and CQ did not change or only slightly decreased against the LM-resistant strain. Assuming that the mechanism of LM resistance is similar in *P. falciparum* and *P. berghei*, these results would suggest a high efficacy of PQ against LM-resistant strains in *P. falciparum*.

It is very interesting to note the activity of LM against PQ-resistant line decreased by 97-fold, a rate which is even higher than its activity against the LM-resistant parasite line selected after 2 years of LM pressure (I_{90} of 64). Thus, the selection of PQ-resistance is associated with a higher level of LM resistance, while, as discussed earlier, the selection of LM resistance is associated with PQ-susceptibility. This demonstrates that 2 different LM-resistance phenotypes exist. The first phenotype is associated with PQ-resistance, while the second is associated with PQ- susceptibility. Assuming that the same pattern prevails in *P. falciparum*, the use of either drug could be associated with resistance or susceptibility to the other. For instance, currently, Coartem® is being used to treat malaria, thus the selection of resistance to LM could be associated with susceptibility to PQ (component of Artekin®). While if Artekin® is first used, resistance to this drug may render Coartem® ineffective.

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Our data show significant 3- and 5-fold decreases in dihydroartemisinin activity against PQ- and LM- resistant strains respectively, an indication of the existence of a slight cross resistance between LM and artemisinin, and PQ and artemisinin, in *P. berghei*. In *P. falciparum*, resistance to the arylamino-alcohol mefloquine, as the result of the increase copy number of *pfmdr*1, is associated with a decrease in activity of artemisinin derivatives (Nelson et al., 2005; Pickard et al., 2003; Price et al., 1999), thus a similar phenomenon may prevail in *P. berghei* with the arylamino-alcohol LM. We report cross resistance between PQ and artemisinin in *P. berghei*, in agreement with previous work (Li 1985; Li et al., 1985).

Thus, if the mechanism of LM and PQ-resistance is similar in *P. berghei* and *P. falciparum*, the selection of LM- and PQ-resistance would be associated with a reduced artemisinin derivative efficacy, compromising the potential of artemisinin based combinations. Consequently, there is an urgent need to clarify the mechanism of LM and PQ-resistance and establish the extent of cross resistance between these important antimalarials. The existence of cross-resistances to chemically and mechanistically unrelated drugs suggests the likely involvement of changes in drug accumulation, i.e. a 'multi-drug resistance' phenotype.

The LM and PQ resistant parasite lines have been selected so as to study the mechanism of drug resistance in *P. berghei* and use this information as platform to explore the resistance mechanism in *P. falciparum*. In the latter species, reports indicate that the use of LM + ATM (Coartem®) selects for 2 haplotypes at 86Y-184Y-1246Y and 86Y-184F-1246D of *pfmdr*1, a gene associated with changes in susceptibility to chloroquine (Dokomajilar et al., 2006; Humphreys et al., 2007; Sisowath et al., 2007; Sisowath et al., 2005). The copy number of *pfmdr*1 has also 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* lumefantrine susceptibility in laboratory selected parasites (Duraisingh and Cowman 2005; Sidhu et al., 2006). These observations indicate that *pfmdr*1 will likely

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contribute to LM resistance, but the full definition of the mechanism of resistance remains to be elucidated since overall, Coartem® retains sensitivity against CQ-resistant isolates. In *P. berghei* and *P. chabaudi*, amplification of the *pfmdr*1 orthologue is associated with mefloquine resistance (Carlton et al., 2001) as in *P. falciparum* (Cowman et al., 1994; Peel 2001; Sidhu et al., 2005). Thus, *pfmdr*1 could also be involved in LM resistance in *P. berghei*.

PQ is a bis-chloroquine derivative (Davis et al., 2005a; Raynes 1999). Thus one could expect that PQ and CQ would share the same mode of action and perhaps a similar mechanism of resistance. However, PQ remains active against CQ-resistant isolates (Basco and Ringwald 2003), clearly indicating that though PQ is closely related to CQ, these 2 drugs have different mechanisms of resistance. To date, no gene or candidate gene has been associated with PQ resistance in *P. falciparum*. Thus, further analysis of this PQ resistant *P. berghei* line could provide insight into the mechanism of PQ resistance.

However, we are aware that mechanism of resistance in *P. falciparum* and murine *Plasmodium* species may be different. For instance, the mechanisms of resistance to CQ are different in *P. falciparum* and in murine malaria and there is still a debate whether those of artemisinin derivatives will be similar (Afonso et al., 2006; Carlton et al., 2001; Hunt et al., 2007; Hunt et al., 2004a; Hunt et al., 2004b). However, for drugs such as mefloquine, antifolates, and atovaquone, similar mechanisms of resistance have been reported (Carlton et al., 2001). Thus, the use of murine malaria could provide critical information on the mechanisms of resistance to PQ and LM.

In summary, we have selected LM and PQ resistant lines of *P. berghei*. The stability of this phenotype indicates that mechanisms that underlie it are coded into the cell genome. Amplification of *pfmdr*1 has been associated with resistance to mefloquine, an amino-alcohol (Carlton et al., 2001). We hypothesise that the same could prevail in LM, which is

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also an amino alcohol. Studies are underway to explore the mechanisms of resistance to LM and PQ.

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Tables

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Table I. Selection of piperaquine resistance in *Plasmodium berghei* GFP ANKA strain using serial technique. Data are presented as effective doses that reduce parasitaemia by 50% and 90% ($ED_{50,}$ ED_{90}) and as 50% and 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).

Passage No	*ED_{50} (mg/kg)*	*(I_{50})*	*ED_{90} (mg/kg)*	*(I_{90})*
Parent	1.30	1	3.52	1
4th	160.28	123.29	262.59	74.70
(6 months cryopreservation) (5th passage)	7.50	5.77	21.90	6.22
9th	21.40	16.46	64.50	18.32
17th	122.00	93.85	194.00	55.11
27th	168.08	129.29	242.38	68.86
Drug free passages				
5th	185.27	142.52	283.71	80.60
10th	191.46	147.28	294.98	83.80
27th passage line after 4 months cryopreservation	110.03	84.64	223.15	63.39

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Table II. Selection of lumefantrine resistance in *Plasmodium berghei* GFP-Luciferase ANKA strain using serial technique. Data are presented as effective doses that reduce parasitaemia by 50% and 90% ($ED_{50,}$ ED_{90}) and as 50% and 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).

Passage No	*ED_{50} (mg/kg)*	*(I_{50})*	*ED_{90} (mg/kg)*	*(I_{90})*
Parent	1.67	1	3.93	1
4^{th}	1.34	0.80	3.49	0.89
12^{th}	1.58	0.95	3.25	0.83
20^{th}	2.96	1.77	5.25	1.34
28^{th}	9.76	5.84	25.50	6.49
36^{th}	42.50	25.33	69.70	17.74
48^{th}	140.15	83.92	249.75	63.55
Drug free passages				
10^{th}	133.17	79.74	256.21	65.19
48^{th} line after 2 months cryopreservation	116.34	69.66	204.58	52.06

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Table III. Response of piperaquine resistant *Plasmodium berghei* GFP ANKA line to amodiaquine (AQ), chloroquine (CQ), lumefantrine (LM) and dihydroartemisinin (DHA). Results 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 sensitive, parent strain).

Antimalarial drug	*ED_{90}* (mg/kg)*		*Index of resistance (I_{90})*
	Parent strain	Resistant line	
AQ	3.72	13.48§	3.62
LM	2.52	245.06¶	97.25
CQ	3.57	26.24‡	7.35
DHA	4.08	12.06§	2.96

*Differences between parent and resistant lines were significant according to Student's t-test: ‡, $p<0.01$; §, $p<0.001$; ¶$p<0.0001$.

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Table IV. Response of lumefantrine resistant *Plasmodium berghei* GFP-Luciferase ANKA line to amodiaquine (AQ), chloroquine (CQ) and dihydroartemisinin (DHA). Results 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 sensitive, parent strain).

Antimalarial drug	*ED_{90}*(mg/kg)*		*Index of resistance (I_{90})*
	Parent strain	Resistant line	
AQ	4.29	4.53†	1.06
PQ	3.70	3.37†	0.91
CQ	4.47	7.22¶	1.62
DHA	6.69	35.86‡	5.36

*Differences between parent and resistant lines were analyzed by Student's t-test: †, $p>0.05$ (insignificant); ‡, significant at $p<0.05$; ¶, significant at $p<0.0001$.

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Figure

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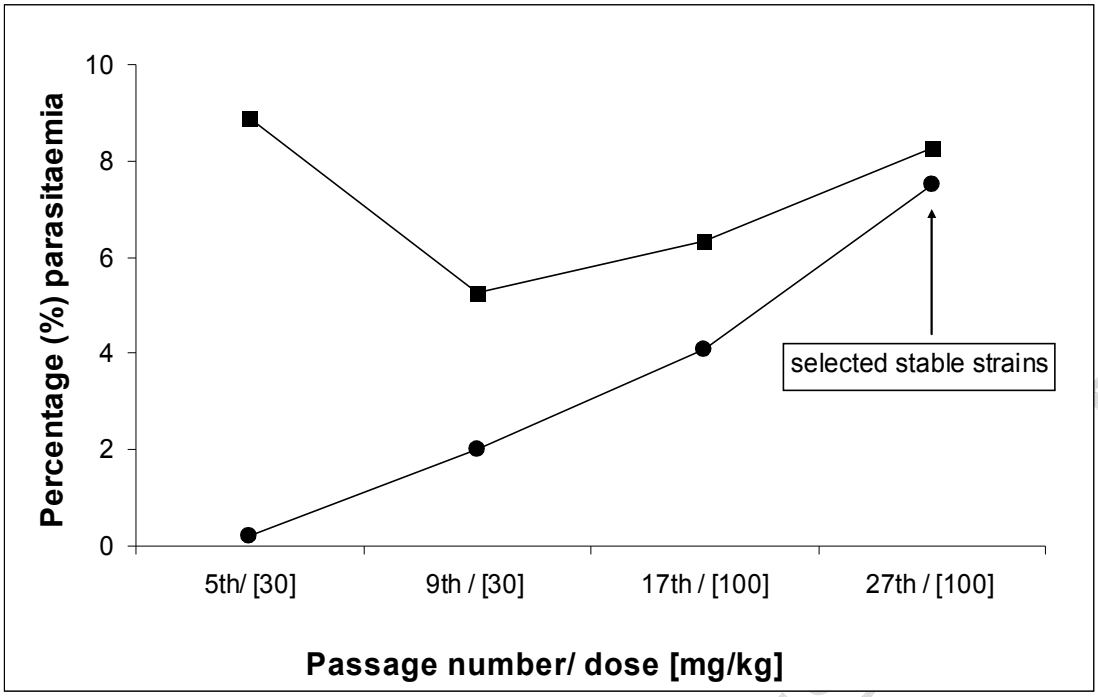
A



B

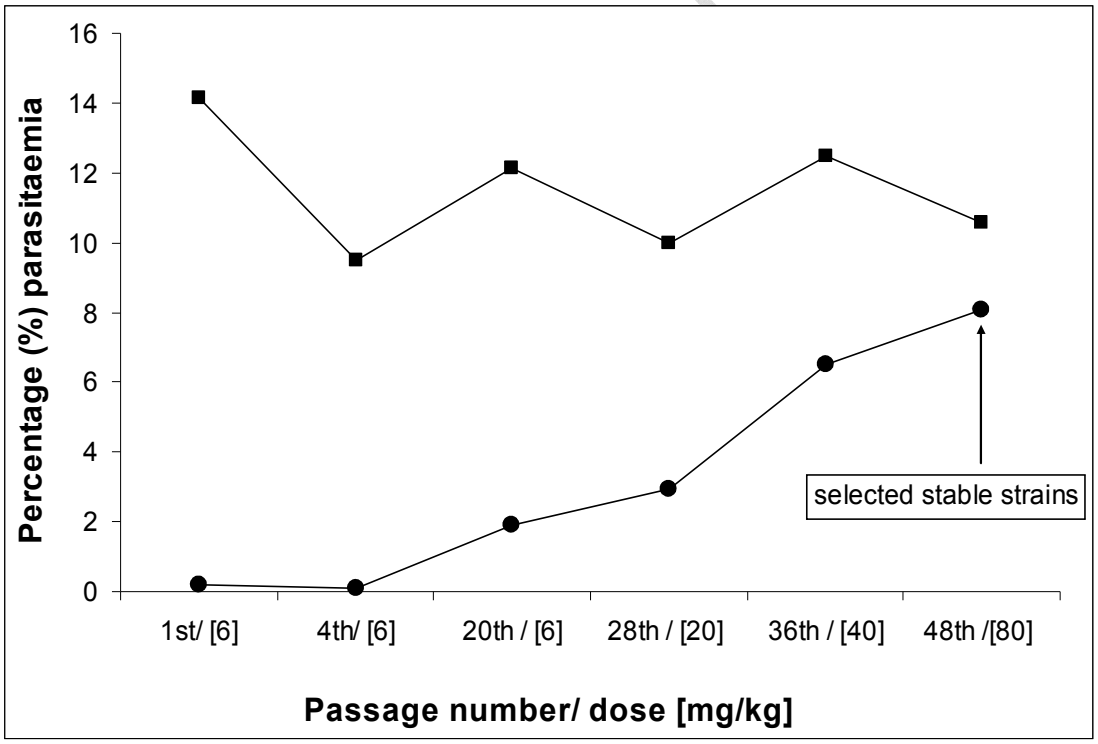


Fig. 1. Development of parasitaemia in the treated (●) and untreated mice (▪) at different levels during the selection of piperaquine (A) and lumefantrine (B) resistant *Plasmodium berghei* GFP-Luciferase ANKA strains in mice. Parasitaemias were assessed after 4 days post infection (in both control and treated groups) and mice were treated using a 4-day test (4-DT, see material and method).