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Within-Host Selection of Drug Resistance in a Mouse Model of Repeated Incomplete Malaria Treatment: Comparison between Atovaquone and Pyrimethamine

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The evolutionary selection of malaria parasites within individual hosts is an important factor in the emergence of drug resistance but is still not well understood. We have examined the selection process for drug resistance in the mouse malaria agent *Plasmodium berghei* and compared the dynamics of the selection for atovaquone and pyrimethamine. Resistance to these drugs has been shown to be associated with genetic lesions in the dihydrofolate reductase gene in the case of pyrimethamine and in the mitochondrial cytochrome *b* gene for atovaquone. A mouse malaria model for the selection of drug resistance, based on repeated incomplete treatment (RICT) with a therapeutic dose of antimalarial drugs, was established. The number of treatment cycles for the development of stable resistance to atovaquone (2.47 ± 0.70; *n* = 19) was found to be significantly lower than for pyrimethamine (5.44 ± 1.46; *n* = 16; *P* < 0.0001), even when the parental *P. berghei* Leiden strain was cloned prior to the resistance selection. Similar results were obtained with *P. berghei* Edinburgh. Mutational changes underlying the resistance were identified to be S110N in dihydrofolate reductase for pyrimethamine and Y268N, Y268C, Y268S, L271V-K272R, and G280D in cytochrome *b* for atovaquone. These results are consistent with the rate of mitochondrial DNA mutation being higher than that in the nucleus and suggest that mutation leading to pyrimethamine resistance is not a rare event.

Drug-resistant parasites have become a major challenge to malaria control today. The emergence of resistance is the outcome of two related processes: the genetic event that produces resistant mutants within individual hosts and the spread of resistance in populations. While, as the initial event in the emergence of resistance, the evolutionary selection of malaria parasites within an individual host is critical, it is still not well understood.

The study of within-host selection of drug resistance benefits from animal models of malaria infection, as it allows genetic and physiological manipulations *in vivo*. Mutations can be selected without mosquito passage (i.e., without meiotic recombination) by exposure of large numbers of malaria parasites to certain drug concentrations. Drug-resistant *Plasmodium falciparum* organisms have been isolated in *in vitro* cultures (1–3), but drug-resistant *Plasmodium berghei*, *Plasmodium yoelii*, and *Plasmodium chaubadi* can be isolated *in vivo* in mice. Two general approaches to study *in vivo* antimalarial resistance selection have been employed and are compared by Peters (4): the serial technique (ST), in which drug dose is gradually increased after each passage, and the 2% relapse technique (2%RT), in which a single and high drug dose is administered at the time of each passage. While these approaches have proven to be effective in the selection of stable resistant strains, both are unnatural treatment regimens and thus might not be suitable models for the study of within-host emergence of antimalarial drug resistance.

More recently, we have introduced an alternative approach for the isolation of atovaquone-resistant strains of *P. berghei* (5) based on cycles of incomplete treatment with a constant therapeutic dose of the antimalarial drug, which mimics treatment failure in the human field situation. In this study, we have developed this approach further into a simple and repeatable animal model (repeated incomplete treatment [RICT]), used the animal model to investigate the process of within-host selection of drug resistance, and show its utility in comparing the development of resistance against two antimalarial agents, atovaquone and pyrimethamine. Pyrimethamine is an antimalarial whose biochemical mechanism of action as an inhibitor of the dihydrofolate reductase (*dhfr*) gene is well understood: resistance to this drug is associated with genetic lesions in the nuclear *dhfr* gene (6, 7). Atovaquone's mechanism of action as an inhibitor of mitochondrial respiratory complex III is similarly well established: resistance is associated with genetic lesions in the mitochondrial cytochrome b (*cytb*) gene (2, 5, 8–13).

MATERIALS AND METHODS

Drugs. Atovaquone, 2-{*trans*-4(4′-chlorophenyl) cyclohexyl}-3-hydroxy-1,4-naphtoquinone, was kindly provided by Mary Pudney of the Wellcome Research Laboratories, United Kingdom, and pyrimethamine was purchased from Sigma-Aldrich Chemie GmbH, Steinheim, Germany. Atovaquone was dissolved in dimethyl sulfoxide (DMSO) and pyrimethamine in 1% glacial acetic acid, and the drugs were stored

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as stock solutions at −20°C and diluted to the required concentration with water freshly before used.

Malarial parasite and mice. *P. berghei* ANKA strains Leiden (obtained from Andy Waters, Leiden University Medical Center, Netherlands) and Edinburgh (obtained from Agnes Kurniawan, Department of Parasitology, Faculty of Medicine, University of Indonesia) were maintained by serial blood passage in 10- to 12-week-old BALB/c mice. Pathogen-free BALB/c mice were obtained from the Animal Resources Centre, Murdoch, Western Australia. During the study, the mice were maintained in the pathogen-free animal house facility of the Eijkman Institute. To ensure that *P. berghei* stocks were free from other pathogens, blood samples from mice inoculated with the stocks, 7 days (plasma) and 15 days or more (dried blood spots on Opti-Spot strips) after inoculation, were screened, respectively, for the presence of mouse hepatitis virus (MHV) and Sendai virus RNA (Eijkman Institute Emerging Virus Research Unit) and serologically for EDIM (epizootic diarrhea of infant mice) virus, *Mycoplasma pulmonis*, MHV, murine norovirus, mouse parvovirus, minute virus of mice, Theiler's murine encephalomyelitis virus (TMEV), and Sendai virus (Idexx BioResearch, Columbia, MO). In all cases, the results were found to be negative.

For experiments requiring fresh clones of the wild-type strain of *P. berghei*, clones were obtained by serial limiting dilution of infected red blood cells and intraperitoneal inoculation of 1 to 10 parasitized red blood cells into BALB/c mice. This study was approved by the institutional review board of the Eijkman Institute (EIREC no. 41).

Monitoring of infections. Peripheral blood smears were prepared daily from tail vein bleeds. The thin films were fixed in methanol (3 min) and then stained with 10% Giemsa. The parasitemia level was determined under 100× oil immersion light microscopy of at least 5,000 red blood cells.

RICT for selection of antimalarial drug-resistant *P. berghei*. The repeated incomplete treatment (RICT) procedure was developed from a method we previously used for the isolation of an atovaquone-resistant mutant of *P. berghei* (5), by standardizing the various steps in the treatment cycles. Parasites were exposed to therapeutic doses of atovaquone (14.4 mg kg^{-1} of body weight) and pyrimethamine (1.5 mg kg^{-1} of body weight) by intraperitoneal injection daily. *P. berghei* was inoculated into BALB/c mice with approximately 10^6 parasitized red blood cells/mouse. Treatment was initiated at parasitemia levels of 3 to 5% and interrupted every time the parasitemia level was reduced to <0.4%, allowing recovery of parasitemia levels in the absence of the drug, before another treatment cycle. This incomplete regime was repeated for several cycles until resistance was observed, indicated by increasing parasitemia levels during treatment. The stability of resistance phenotypes was established by passage to new mice and challenges with therapeutic doses of the antimalarials.

Determination of mutations in the *cytb* and *dhfr* genes. Approximately 50 μl of infected mouse tail blood was collected and stored at −20°C in 1.5-ml heparinized Eppendorf tubes. Parasite DNA was isolated from saponin (Sigma-Aldrich, St. Louis, MO)-lysed blood by treatment with Chelex 100 (Sigma-Aldrich, St. Louis, MO) essentially as described by Wooden et al. (14). DNA was used immediately for PCR or stored at −20°C.

Fragments of the mitochondrial *cytb* gene were amplified by PCR as described previously (5), employing primer pair 5′-CCTTTAGGGTATG ATACAGC and 5′-GTTTGCTTGGGAGCTGTAATC (for the Qo_2 domain) or 5′-TGCCTAGACGTATTCCTGAT and 5′-TGATGTATCATA CCCTAAAG (for the Qo_1 domain). PCR was carried out for 34 cycles of denaturation at 94°C for 15 s (first cycle 5 min), annealing at 55°C (first primer pair) or 52°C (second primer pair) for 15 s, and extension at 72°C for 2 min (final extension, 5 min). To amplify *dhfr* gene fragment 99-316, primers 5′-GCAATATGTGCATGTTGTAAAGT and 5′-TTTTGGGAAT ACTTTCCGAA were employed. PCR was run for 29 cycles of denaturation at 94°C for 30 s (first cycle, 5 min), annealing at 50°C for 30 s, and extension at 65°C for 2 min (final extension, 5 min). The PCR products obtained were directly sequenced using the forward primers to generate sequences in an ABI 377 automatic sequencer. The sequences were aligned using the BioEdit program.

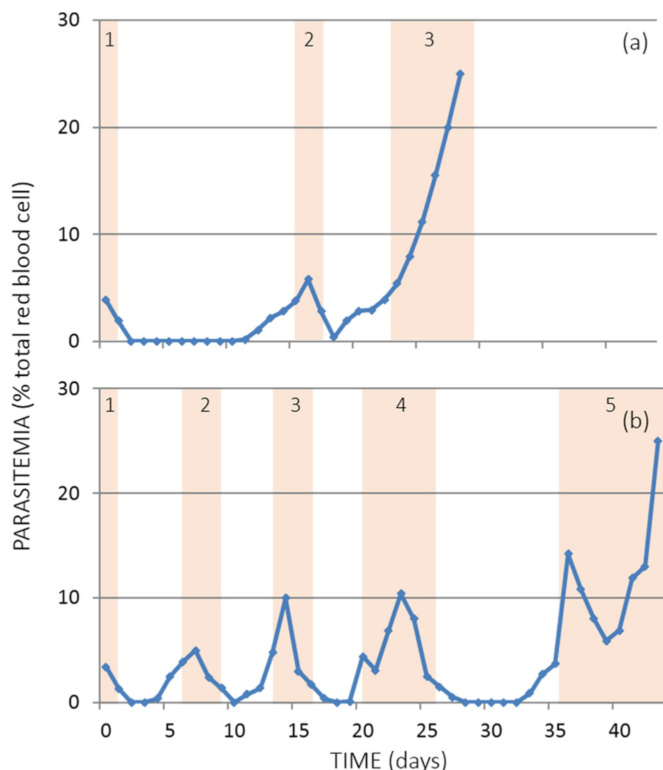

FIG 1 Development of resistance to atovaquone (a) and pyrimethamine (b) following repeated incomplete treatment (RICT) of *P. berghei* Leiden in mice. Shaded areas indicate treatment periods. A stable resistance phenotype was observed in the third and fifth treatment cycles for atovaquone and pyrimethamine, respectively.

RESULTS

Rapid development of resistance during repeated incomplete malaria treatment. The features of the experimental system developed in the present study are shown in Fig. 1 The experimental system is based on cycles of incomplete treatment of *P. berghei*-infected mice with a therapeutic dose of an antimalarial drug, i.e., interruption of treatment every time parasitemia decreases to a level below 0.4% and allowing recovery of parasitemia before starting a new cycle of treatment. In a typical experiment (Fig. 1a), a stable resistance phenotype was already observed in the third treatment with atovaquone. Rapid development of resistance was also observed for pyrimethamine, although this required five cycles of treatment (Fig. 1b).

To establish the generality and reproducibility of the above-described observation, we repeated the experiments with larger numbers of mice. Table 1 summarizes the treatment and recovery times observed during the treatment of each *P. berghei*-infected mouse with atovaquone and pyrimethamine. The average number of treatment cycles for the development of a stable phenotype of resistance to atovaquone was found to be 2.67 ± 0.87 (mean ± standard deviation [SD]; $n = 9$), significantly faster than that to pyrimethamine (5.33 ± 1.32; $n = 9$; $P < 0.0001$) (Table 2).

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TABLE 1 Rapid development of resistance to atovaquone and pyrimethamine of uncloned and cloned parasites

Type of parasites	Drug[a]	Isolate	No. of days of treatment-no. of days of recovery for cycle[b]: 1	2	3	4	5	6	7	8	No. of cycles to resis-tance[c]
Uncloned	ATV	PbLASN1	2-13	3-4	18-●						3
		PbLASN2	2-12	18-●							2
		PbLASN3	2-6	10-●							2
		PbLASN4	3-8	8-●							2
		PbLASN5	3-9	6-●							2
		PbLASN6	2-10	2-●							2
		PbLASN7	1-12	4-20	3-3	2-●					4
		PbLSJ1	1-7	3-13	2-18	3-●					4
		PbLSJ2	3-18	1-32	5-●						3
	PYR	PbLPSN1	2-4	4-3	4-3	6-9	6-●				5
		PbLPSN2	2-16	2-2	5-●						3
		PbLPSN3	3-3	4-3	6-9	2-3	6-●				5
		PbLPSN4	2-7	2-5	2-5	4-●					4
		PbLPSN5	1-7	2-10	1-8	3-15	3-●				5
		PbLPSN6	1-7	1-11	1-12	1-14	2-18	3-●			6
		PbLPSN7	1-4	2-10	1-14	1-14	2-20	2-36	5-●		7
		PbLPSN8	1-7	1-11	2-11	1-15	3-12	1-22	4-●		7
		PbLPSN9	1-7	1-11	1-12	1-15	1-30	6-●			6
Cloned	ATV	PbLACSN1	2-8	20-●							2
		PbLACSN2	3-8	19-●							2
		PbLACSN3	2-8	20-●							2
		PbLACSN4	2-9	16-3	8-●						3
		PbLACSN5	2-7	4-3	20-●						3
		PbLACSN6	2-13	20-●							2
		PbLACSN7	2-10	15-●							2
		PbLACSN8	2-9	23-●							2
		PbLACSN9	2-17	2-17	3-●						3
		PbLACSN10	2-27	22-●							2
	PYR	PbLPCSN1	2-4	2-2	2-2	9-3	2-3	9-●			6
		PbLPCSN2	2-4	2-2	2-2	13-4	2-8	4-2	14-●		7
		PbLPCSN3	2-4	2-8	14-●						3
		PbLPCSN4	2-3	2-3	2-3	2-5	2-6	2-25	3-3	17-●	8
		PbLPCSN5	1-7	2-3	2-3	10-15	5-●				5
		PbLPCSN6	2-4	2-6	3-4	8-8	7-●				5
		PbLPCSN7	2-3	2-4	3-7	2-6	8-4	17-●			6

[a] ATV, atovaquone; PYR, pyrimethamine.
[b] No. of days of treatment to bring the parasitemia level down to below 0.4%-no. of days of recovery for the parasitemia level to return to 3 to 5%. ●, termination of treatment cycles due to the development of stable resistance.
[c] The numbers of treatment cycles that led to resistance were significantly different between atovaquone and pyrimethamine; $P < 0.0001$ in both uncloned and cloned parasites.

The rapid development of drug resistance is not due to a high number of preexisting mutants. To ensure that the rapid appearance of antimalarial-resistant mutants was not an anomaly due to an unusually high number of preexisting mutant cells accumulated during previous growth and passages of the laboratory *P. berghei* strain, we cloned wild-type *P. berghei*. The development of atovaquone and pyrimethamine resistance was then examined using the clones isolated. The numbers of treatment cycles leading to resistance after cloning were 2.30 ± 0.48 for atovaquone ($n = 10$) and 5.57 ± 1.72 for pyrimethamine ($n = 7$), which in both cases are statistically the same as those before cloning. The combined data of the before and after cloning experiments provide strong

TABLE 2 Rapid development of *P. berghei* resistance to atovaquone and pyrimethamine by RICT

Type of parasites	Atovaquone: No. of isolates	No. of cycles[a]	No. of days[a]	Pyrimethamine: No. of isolates	No. of cycles[a]	No. of days[a]
Uncloned	9	2.67 ± 0.87	33.33 ± 17.1	9	5.33 ± 1.32	60.56 ± 30.8
Cloned	10	2.30 ± 0.48	34.90 ± 7.1	7	5.57 ± 1.72	51.43 ± 17.6
Total	19	2.47 ± 0.70[b]	34.16 ± 12.4	16	5.44 ± 1.46[b]	56.56 ± 25.5

[a] Means ± standard deviations.
[b] $P < 0.0001$.

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TABLE 3 Mutations underlying the atovaquone and pyrimethamine resistance of *P. berghei*

Drug	Mutation(s)	No. of isolates	Isolate(s)
Atovaquone	Y268S	1	PbLACSN4
	Y268N	4	PbLSJ1, PbLASN4, PbLACSN5, PbLACSN7
	Y268C	6	PbLASN1, PbLASN2, PbLASN3, PbLASN7, PbLACSN1, PbLACSN2
	L271V + K272R	7	PbLSJ2, PbLASN5, PbLASN6, PbLACSN3, PbLACSN6, PbLACSN8, PbLACSN10
	G280D	1	PbLACSN9
Pyrimethamine	S110N	16	PbLPSN1 to -9, PbLPCSN1 to -7

evidence of the statistical significance of the finding that the number of treatment cycles for the development of stable resistance to atovaquone (2.47 ± 0.70; $n = 19$) is faster than that to pyrimethamine (5.44 ± 1.46; $n = 16$; $P < 0.0001$) (Table 2).

Mutations responsible for the resistance to atovaquone and pyrimethamine. The mutations that underlie the resistance to the antimalarial drugs are shown in Table 3. Seven mutational changes affecting the cytochrome *b* protein of respiratory complex III were found to be associated with the resistance to atovaquone, three of which were single amino acid changes affecting residue 268 in the Qo_2 domain of the protein, Y268N, Y268C, and Y268S, in 11 of 19 resistant isolates. A pair of double mutations affecting the Qo_2 domain, L271V-K272R, was found in 7 isolates. One resistant isolate had a single amino acid change affecting residue 280 (G→D) in the sixth transmembrane domain. All 16 pyrimethamine-resistant *P. berghei* isolates carry DHFR mutation S110N, convergent to the most common mutation in *Plasmodium falciparum*.

The rapid development of resistance to atovaquone and pyrimethamine is observed in different strains. To confirm that the rapid development of resistance following RICT is not an unusual property of *P. berghei* strain Leiden, we have repeated the experiments employing the Edinburgh strain (Table 4). The number of treatment cycles for stable resistance to atovaquone to develop was 3.75 ± 0.96 for *P. berghei* Edinburgh, higher than that for *P. berghei* Leiden (2.47 ± 0.70; $P = 0.005$). In the case of pyrimethamine, the number of treatment cycles for resistance to develop was found to be higher, 8.25 ± 1.50 for *P. berghei* Edinburgh, compared to 5.44 ± 1.46 for *P. berghei* Leiden ($P = 0.003$). Similar to the case with *P. berghei* Leiden, however, the number of treatment cycles for stable resistance to atovaquone to develop in *P. berghei* Edinburgh was significantly lower than for pyrimethamine ($P = 0.0023$). Only one new atovaquone resistance mutation, L271F-V284F, was found in *P. berghei* Edinburgh; the other resistance mutations corresponded to those in the Leiden strain. All pyrimethamine-resistant *P. berghei* Edinburgh isolates carried the DHFR mutation S110N.

DISCUSSION

While the previously reported ST and 2%RT procedures have proven to be effective in the selection of stable resistant strains in mice, a mouse model that better reflects malaria treatment failure in the human field situation is needed for the elucidation of within-host emergence of antimalarial drug resistance. The new mouse malaria model we have developed for this purpose, based on repeated incomplete treatment (RICT) of *P. berghei* infection, proved to be reproducible in raising resistant mutants against atovaquone and pyrimethamine. Moreover, our results suggest that RICT is a more efficient method for the isolation of resistant mutants for both antimalarial drugs. RICT of *P. berghei* leads to rapid development of phenotypically stable resistance to atovaquone in only 2.5 treatment cycles (approximately 20 days), which is significantly faster than the ST method, which required more than 5 months for the same strain of *P. berghei* (9). We showed that our finding is not due to a high number of preexisting mutant cells accumulated during previous growth and passages and is not restricted to a single strain, as similar results were obtained for *P. berghei* strains Leiden and Edinburgh.

Surprisingly, the RICT method has also allowed the establishment of *P. berghei* stable strains resistant to pyrimethamine in only 5 cycles (about 37 days), compared to 50 weeks required by the ST method (15). In addition to being both a more natural and more efficient method to select resistant mutants *in vivo*, the RICT approach also requires considerably fewer experimental mice than the ST and 2%RT techniques. In ST and 2%RT, infected blood needs to be passaged to naive mice for each new drug challenge, while the RICT method selects resistant *P. berghei* mutants in single mouse hosts.

It is important to consider the potential role of immunity to the parasite as a cofounding factor during the length of repeated incomplete treatments in RICT. The acquisition of immunity to *P. berghei* in mice is well documented. Thus, while primary *P. berghei* infections in white mice invariably resulted in 100% mortality, a second infection after cure with an antimalarial drug is usually milder, as characterized by the survival of the majority of infected mice (16–18). The manifestation of such acquired immunity to *P. berghei*, however, was not observed in our RICT model. Even in the case of pyrimethamine treatments, where resistant parasites

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TABLE 4 Mutations underlying atovaquone and pyrimethamine resistance in *P. berghei* Edinburgh

Atovaquone			Pyrimethamine		
Isolate	No. of cycles to resistance	Mutation	Isolate	No. of cycles to resistance	Mutation
PbEAJR1	3	L271V + K272R	PbEPSN1	7	S110N
PbEAJR2	4	L271V + K272R	PbEPSN2	7	S110N
PbEAJR3	3	L271V + V284F	PbEPSN3	10	S110N
PbEASN1	5	L271F + V284F	PbEPSN4	9	S110N
Mean ± SD	3.75 ± 0.96[a]			8.25 ± 1.50[a]	

[a] $P = 0.0023$.

emerged only after 5 to 7 incomplete treatments, the recovery of the parasitemia level to 3 to 5% between each treatment was not affected by the length of treatment. This feature is consistent with a previous observation that infection caused by *P. berghei* strains isolated from relapsed infections of white mice caused significantly greater mortality than did the parent strains when used to reinfect cured mice (19). This observation indicates that acquired immunity to *P. berghei* does not recognize relapsed strains, which might be selected variants with different antigenic characteristics.

The finding that the development of stable resistance to atovaquone is significantly faster than for pyrimethamine is consistent with the general observation that the rate of mutation in the mitochondrial DNA (mtDNA) is significantly higher than that of the nuclear DNA; the mutation rate of animal mtDNA is 5 to 10 times faster than that of single-copy nuclear DNA (20), including for the mitochondrion-encoded cytochrome *b* (21). Comparison between genetic lesions that underlie the resistance phenotype in mutants isolated by the RICT and ST/2%RT methods could provide insight into the mechanism of mutant selection. In *Plasmodium* spp., 19 mutations encoded by the mitochondrial cytochrome *b* gene—I125M, M133I, T142I, L144F/S, L171F, I258M, F267I, Y268C/N/S, L271F/V, K272R, P275T, G280D, L283I, and V284K/F—have been documented to be associated with atovaquone resistance (1, 5, 8–11). Nine of these mutations are located in the Qo_2 domain of the cytochrome *b* protein; only four mutations (M133I, T142I, and L144F/S) are in the Qo_1 domain, and the rest (I125M, L171F, G280D, L283I, and V284K/F) are in the third, fourth, and sixth transmembrane domains. Many of these mutations have been shown to be associated with *in vitro* resistance to atovaquone. For *P. berghei*, the degrees of resistance conferred by some mutations—M133I, L144S, Y286C/N, L271V, K272R, and V284F—have been tested directly by measuring the activity of dihydroorotate (DHO)-cytochrome *c* reductase in isolated mitochondria. The 50% inhibitory concentration (IC_{50}) of atovaquone-resistant mutants is much higher (1.5 up to 40 nM) than for the atovaquone-sensitive clones (0.132 to 0.465 nM), with the highest associated with mutants carrying Y268C and Y268N mutations (13).

Atovaquone treatment failure of *P. falciparum* in humans is only associated with mutations in codon 268: either Y268S (10, 22–25), Y268N (26), or Y268C (12). Thus, it is of interest that more than half of atovaquone resistance mutations observed in the RICT study are also in codon 268 of the cytochrome *b* Qo_2 domain (11 out of 19 resistant isolates) and that all three *P. falciparum* codon 268 mutations were observed in the *P. berghei* resistant isolates. Y268S is of particular interest, as this common atovaquone-resistant mutation of *P. falciparum* (12) has not been reported for *P. berghei* previously. The other *P. berghei* resistant isolates carry mutations in the neighboring codon 271 (L271V/F), which is always associated with K272R or V284F mutations. All of these mutations are also located in the Qo_2 domain of the cytochrome *b* gene, except V284F, in the sixth transmembrane domain, close to Qo_2 domain. The ubiquinol oxidation (Qo_2) domain of cytochrome *b* is responsible for charge separation caused by the electron transfer to the iron-sulfur protein, ultimately resulting in proton translocation. Amino acid residues Y268 and L271 are highly conserved, and our results further confirm the importance of these residues in the binding of atovaquone to the catalytic Qo site (27).

All our pyrimethamine-resistant *P. berghei* isolates were found to carry the S110N mutation, convergent with S108N in *P. falciparum*. Most pyrimethamine-resistant field isolates of *P. falciparum* are associated with the S108N mutation, but multiple mutations affecting codons 51, 59, and 164, associated with higher degrees of resistance, have also been observed (28). The constant therapeutic dose employed in the present study appears to predispose toward resistance mutations at S110/S108.

The small number of treatment cycles required for the development of stable resistance to atovaquone and pyrimethamine in RICT experiments is consistent with the history of *P. falciparum* resistance to atovaquone, as well as pyrimethamine, in the population. The finding in an early field trial of atovaquone (29) that irrespective of the duration of therapy (overall cure rates were approximately 67%), there was a marked decrease in susceptibility to the antimalarial in the recrudescent parasites after the first treatment, is consistent with the fast phenotypic detection of resistance to atovaquone in RICT (in many cases in one cycle, or 8 days). Resistance had developed rapidly, in approximately 1 year, from the introduction of pyrimethamine to the population in 1967 (30). RICT could be developed as a useful tool to predict the potential emergence of resistance, not only to current less understood antimalarials but also to newly introduced compounds, providing knowledge essential for planning malaria control and devising strategies to delay the emergence of resistance.

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REFERENCES

1. **Rathod PK, McErlean T, Lee PC.** 1997. Variations in frequencies of drug resistance in *Plasmodium falciparum*. Proc Natl Acad Sci U S A **94:**9389–9393.
2. **Korsinczky M, Chen N, Kotecka B, Saul A, Rieckmann K, Cheng Q.** 2000. Mutation in *Plasmodium falciparum* cytochrome *b* that are associated with atovaquone resistance are located at a putative drug-binding site. Antimicrob Agents Chemother **44:**2100–2108. http://dx.doi.org/10.1128/AAC.44.8.2100-2108.2000.
3. **Nzila A, Mwai L.** 2010. In vitro selection of *Plasmodium falciparum* drug-resistant parasite lines. J Antimicrob Chemother **65:**390–398. http://dx.doi.org/10.1093/jac/dkp449.
4. **Peters W.** 1999. The chemotherapy of rodent malaria. LVII. Drug combinations to impede the selection of drug resistance, part 1: which model is appropriate? Ann Trop Med Parasitol **93:**569–587.
5. **Siregar JE, Syafruddin D, Matsuoka H, Kita K, Marzuki S.** 2008. Mutation underlying resistance of *Plasmodium berghei* to atovaquone in the quinone binding domain 2 (Qo_2) of the cytochrome *b* gene. Parasitol Int **57:**229–232. http://dx.doi.org/10.1016/j.parint.2007.12.002.
6. **Peterson DS, Walliker D, Wellems TE.** 1988. Evidence that a point mutation in dihydrofolate reductase-thymidylate synthase confers resistance to pyrimethamine in *falciparum* malaria. Proc Natl Acad Sci U S A **85:**9114–9118. http://dx.doi.org/10.1073/pnas.85.23.9114.
7. **Syafruddin D, Asih PBS, Casey GJ, Maguire J, Baird JK, Nagesha HS, Cowman AF, Reeder JC.** 2005. Molecular epidemiology of *Plasmodium falciparum* resistance to antimalarial drugs in Indonesia. Am J Trop Med Hyg **72:**174–181.
8. **Srivastava IK, Morrisey JM, Darrouzer E, Daldal F, Vaidya AB.** 1999. Resistance mutations reveal the atovaquone-binding domain of cytochrome *b* in malaria parasites. Mol Microbiol **33:**704–711. http://dx.doi.org/10.1046/j.1365-2958.1999.01515.x.

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9. **Syafruddin D, Siregar JE, Marzuki S.** 1999. Mutations in the cytochrome *b* gene of *Plasmodium berghei* conferring resistance to atovaquone. Mol Biochem Parasitol **104:**185–194. http://dx.doi.org/10.1016/S0166-6851(99)00148-6.
10. **Schwöbel B, Alifrangis M, Salanti A, Jelinek T.** 2003. Different mutation patterns of atovaquone resistance to *Plasmodium falciparum in vitro* and *in vivo*: rapid detection of codon 268 polymorphisms in the cytochrome *b* as potential *in vivo* resistance marker. Malar J **2:**5. http://dx.doi.org/10.1186/1475-2875-2-5.
11. **Afonso A, Neto Z, Castro H, Lopes D, Alves AC, Tomas AM, Rosario VD.** 2010. *Plasmodium chaubadi chaubadi* malaria parasites can develop stable resistance to atovaquone with a mutation in the cytochrome *b* gene. Malar J **9:**135. http://dx.doi.org/10.1186/1475-2875-9-135.
12. **Musset L, Bouchaud O, Matheron S, Massias L, Le Bras J.** 2006. Clinical atovaquone-proguanil resistance of *Plasmodium falciparum* associated with cytochrome *b* codon 268 mutations. Microbes Infect **8:**2599–2604. http://dx.doi.org/10.1016/j.micinf.2006.07.011.
13. **Siregar JE, Kurisu G, Kobayashi T, Matsuzaki M, Sakamoto K, Mi-ici F, Watanabe Y, Hirai M, Matsuoka H, Syafruddin D, Marzuki S, Kita K.** 2015. Direct evidence for the atovaquone action on the *Plasmodium* cytochrome bc1 complex. Parasitol Int **64:**295–300. http://dx.doi.org/10.1016/j.parint.2014.09.011.
14. **Wooden J, Kyes S, Sibley CH.** 1993. PCR and strain identification in *Plasmodium falciparum*. Parasitol Today **9:**303–305. http://dx.doi.org/10.1016/0169-4758(93)90131-X.
15. **Ferone R.** 1970. Dihydrofolate reductase from pyrimethamine-resistant *Plasmodium berghei*. J Biol Chem **245:**850–854.
16. **Singer I.** 1954. The cellular reactions to infections with *Plasmodium berghei* in the white mouse. J Infect Dis **94:**241–261. http://dx.doi.org/10.1093/infdis/94.3.241.
17. **Box ED, Gingrich WD.** 1958. Acquired immunity to *Plasmodium berghei* in the white mouse. J Infect Dis **103:**291–300. http://dx.doi.org/10.1093/infdis/103.3.291.
18. **Nunes JK, Stambach MN, Wirth DF.** 2009. Secreted antibody is required for immunity to *Plasmodium berghei*. Infect Immun **77:**414–418. http://dx.doi.org/10.1128/IAI.00982-08.
19. **Cox HW.** 1962. The behavior of *Plasmodium berghei* strains isolated from relapsed infections of white mice. J Protozool **9:**114–118. http://dx.doi.org/10.1111/j.1550-7408.1962.tb02591.x.
20. **Brown WM, George M, Jr, Wlson AC.** 1979. Rapid evolution of animal mitochondrial DNA. Proc Natl Acad Sci U S A **76:**1967–1971. http://dx.doi.org/10.1073/pnas.76.4.1967.
21. **Comtamine V, Picard M.** 2000. Maintenance and integrity of the mitochondrial genome: a plethora of nuclear genes in the budding yeast. Microbiol Mol Biol Rev **64:**281–315. http://dx.doi.org/10.1128/MMBR.64.2.281-315.2000.
22. **David KP, Alifrangis M, Salanti A, Vestergaard LS, Ronn A, Bygbjerg IB.** 2003. Atovaquone/proguanil resistance in Africa: a case report. Scand J Infect Dis **35:**897–898.
23. **Schwartz E, Bujanover S, Kain KC.** 2003. Genetic confirmation of atovaquone-proguanil-resistant *Plasmodium falciparum* malaria acquired by a nonimmune traveler to East Africa. Clin Infect Dis **37:**450–451. http://dx.doi.org/10.1086/375599.
24. **Legrand E, Demar M, Volney B, Ekala MT, Quinternet M, Bouchier C, Fandeur T, Rogier C, Carme B, Puijalon O, Esterre P.** 2007. First case of emergence of atovaquone resistance in *Plasmodium falciparum* during second-line atovaquone-proguanil treatment in South America. Antimicrob Agents Chemother **51:**2280–2281. http://dx.doi.org/10.1128/AAC.01532-06.
25. **Fisher N, Majid RA, Antoine T, Al-Helal M, Warman AJ, Johnson DJ, Lawrenson AS, Ranson H, O'Neil PM, Ward SA, Biagini GA.** 2012. Cytochrome *b* mutation Y268S conferring atovaquone resistance phenotype in malaria parasite results in reduced parasite bc_1 catalytic turnover and protein expression. J Biol Chem **287:**9731–9741. http://dx.doi.org/10.1074/jbc.M111.324319.
26. **Fivelman QL, Butcher GA, Adagu IS, Warhurst DC, Pasvol G.** 2002. Malarone treatment failure and *in vitro* confirmation of resistance of *Plasmodium falciparum* isolate from Lagos, Nigeria. Malar J **1:**1. http://dx.doi.org/10.1186/1475-2875-1-1.
27. **Birth D, Kao WC, Hunte C.** 2014. Structural analysis of atovaquone-inhibited cytochrome *bc1* complex reveals the molecular basis of antimalarial drug action. Nat Commun **5:**4029. http://dx.doi.org/10.1038/ncomms5029.
28. **Ménard D, Andriantsoanirina V, Jahevitra M, Barnades C, Tichit M, Bouchier C, Sibley CH.** 2008. Dyhidrofolate reductase I164L mutation in *Plasmodium falciparum*, Madagascar. Emerg Infect Dis **14:**1166–1167. http://dx.doi.org/10.3201/eid1407.071498.
29. **Looareesuwan S, Viravan C, Webster HK, Kyle DE, Hutchinson DB, Canfield CJ.** 1996. Clinical studies of atovaquone, alone or in combination with other antimalarial drugs, for treatment of acute uncomplicated malaria in Thailand. Am J Trop Med Hyg **54:**62–66.
30. **World Health Organization. May** 2014. Emergence and spread of artemisinin resistance calls for intensified efforts to withdraw oral artemisinin-based monotherapy from the market. WHO/HTM/GMP/2014.3. World Health Organization, Geneva, Switzerland.

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