Mutations in Plasmodium falciparum Cytochrome b That Are Associated with Atovaquone Resistance Are Located at a Putative Drug-Binding Site†

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Atovaquone is the major active component of the new antimalarial drug Malarone. Considerable evidence suggests that malaria parasites become resistant to atovaquone quickly if atovaquone is used as a sole agent. The mechanism by which the parasite develops resistance to atovaquone is not yet fully understood. Atovaquone has been shown to inhibit the cytochrome bc1 (CYT bc1) complex of the electron transport chain of malaria parasites. Here we report point mutations in Plasmodium falciparum CYT b that are associated with atovaquone resistance. Single or double amino acid mutations were detected from parasites that originated from a cloned line and survived various concentrations of atovaquone in vitro. A single amino acid mutation was detected in parasites isolated from a recrudescent patient following atovaquone treatment. These mutations are associated with a 25- to 9,354-fold range reduction in parasite susceptibility to atovaquone. Molecular modeling showed that amino acid mutations associated with atovaquone resistance are clustered around a putative atovaquone-binding site. Mutations in these positions are consistent with a reduced binding affinity of atovaquone for malaria parasite CYT b.

The widespread resistance of malaria parasites to standard antimalarial drugs is a serious global health problem. The urgent need for new antimalarial drugs has led to the development of atovaquone (566C80) which, combined with proguanil, has been licensed as Malarone. There is some concern that parasites may develop resistance to Malarone. In one study, 33% of patients treated with atovaquone alone experienced a recrudescence of parasitemia after treatment. These parasites tolerated up to 1,000-fold higher concentrations of atovaquone than did the pretreated parasites (16). Atovaquone-resistant parasites have been readily selected in vitro. Up to 1 in 107 parasites became resistant to the drug after having been cultured in the presence of 10−8 M atovaquone for 5 weeks (21, 23).

Atovaquone has potent blood schizonticidal activity and is also effective against the preerythrocytic (2, 4, 5) and sexual stages (8, 9) of the malaria parasite. It acts by inhibiting mitochondrial electron transport (10) and collapsing mitochondrial membrane potential (25). From these observations and on the basis of its structural similarity to ubiquinol, it has been postulated that atovaquone binds to parasite cytochrome b (CYT b) (31). The inhibitors stigmatellin and 5-n-undecyl-4,7-dioxo-benzoxythiazol (UHDBT), which are structurally similar to atovaquone, have been shown to bind to the ubihydroquinone (Q0) site of CYT b and inhibit electron transport. Single point mutations within the Q0 site confer resistance to these inhibitors in a variety of microorganisms (7). Two mutations in close proximity to the Q0 site in Pneumocystis carinii are associated with atovaquone prophylaxis failure. Atovaquone-resistant Plasmodium yoelii lines have been derived from infected mice treated with suboptimal doses of atovaquone. All resistant lines have single or double amino acid mutations in their CYT b protein sequences, which are close to the predicted Q0 site region. These mutations were associated with increased resistance to the collapsing of mitochondrial membrane potentials and the inhibition of respiration afforded by atovaquone (24). A potential drug-binding cavity has also been identified in the chicken CYT b. A similar study has been performed with Plasmodium berghei, and mutations in CYT b were again associated with atovaquone resistance (27). However, the three-dimensional structure of the Plasmodium falciparum CYT b and atovaquone-binding site has not been studied.

In this report, we describe mutations found in the CYT b gene of atovaquone-resistant P. falciparum and the resulting amino acid changes to the predicted atovaquone-binding site. The crystal structures of the chicken and bovine CYT bc1 complexes (13, 34) and recently found mutations in the parasite CYT b associated with atovaquone resistance (24, 27, 33) have made molecular modeling of the P. falciparum CYT b protein and prediction of the potential atovaquone binding site possible. Our modeling results demonstrate that the amino acids associated with atovaquone resistance in P. falciparum are clustered around the putative atovaquone binding site and that mutations in these positions are likely to reduce the binding affinity of atovaquone for P. falciparum CYT b.

MATERIALS AND METHODS

Parasites. P. falciparum TM93-C1088 was kindly provided by Dennis Kyle, Department of Immunology and Parasitology, U.S. Army Medical Component, Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand. The parasite was isolated from a Thai patient who experienced a recrudescence of parasitemia after treatment with atovaquone (1,000 mg once a day for 3 days) and pyrimethamine (16). P. falciparum K1 is a laboratory line originally isolated from a Thai patient (28). K1-1D4 is a cloned line of K1 produced in the AMI...
In vitro cultivation of P. falciparum. Parasites were maintained in vitro using conditions described by Trager and Jensen (30).

Cloning of the P. falciparum K1 line. K1 parasites were cultured in vitro and cloned once by the method of limiting dilution. Briefly, the number of infected red blood cells in the culture stock was determined by multiplying the number of red blood cells by the percentage of parasitemia. The culture stock was then diluted with medium containing 5% (vol/vol) uninfected red blood cells to concentrations at which the number of infected red blood cells per 100 μl was 3, 1, or 0.3. A 100-μl volume of the diluted cultures was transferred to each well of 96-well plates and cultured for 4 to 5 weeks. When the cultures had reached a 1% parasitemia, cells from the positive wells were transferred to 24-well plates and subsequently to 10- and 50-ml culture flasks. K1-1D4, the parent clone for this study, originated from one positive well on the one-per-well plate where approximately 7% of the wells yielded detectable parasites.

In vitro development of atovaquone resistance in P. falciparum K11D4. Two protocols were used to select atovaquone resistant parasites from the K1-1D4 cloned line of P. falciparum, a step-wise selection and a single-step selection. The procedures are detailed as follows.

(i) Stepwise selection. Stepwise selection (Fig. 1) was carried out as follows. Parasites were initially cultured in one flask and split into four cultures when parasitemia reached approximately 2%. These four cultures were maintained for one cycle until the parasitemia reached approximately 3%. Parasitized red blood cells from one of the four flasks were cryopreserved as the atovaquone-sensitive parents (parent). Parasites in the remaining three flasks were cultured continuously in medium containing 2 × 10⁻⁵ M atovaquone, a concentration equivalent to the 90% inhibitory concentration (IC₉₀) (2.2 × 10⁻⁸ M) for this cloned line of parasites. Culture medium was changed every second day. Cultures were split in a 1:1 ratio approximately every 10 days until the parasitemia reached 0.1%. The parasites that survived were then cultured in two flasks (A and B) and sequentially subjected to 5 × 10⁻⁷, 1.5 × 10⁻⁷, 5 × 10⁻⁸, 1.5 × 10⁻⁸, and 1.5 × 10⁻⁹ M atovaquone. Aliquots of parasites were removed after surviving each drug concentration for cryopreservation and DNA preparation.

(ii) One-step selection. One-step selection was carried out as follows (Fig. 1). Parent parasites were split into nine flasks and were subjected to atovaquone concentration for 6 weeks. The surviving parasites were cryopreserved and extracted for DNA.

Cloning of atovaquone-resistant mutant K1-1D4 parasites. Parasites that survived 2 × 10⁻⁸ M atovaquone initially and 5 × 10⁻⁹ M subsequently were cloned using methods described above.

Cloning of atovaquone-resistant mutant K1-1D4 parasites. Parasites that survived 2 × 10⁻⁸ M atovaquone initially and 5 × 10⁻⁹ M subsequently were cloned using methods described above.

In vitro drug susceptibility tests. Two experiments were carried out using the following two protocols.

(i) The susceptibility of the K1 and TM93-C1088 lines of P. falciparum was determined for atovaquone (Jacobus Pharmaceutical Company, Inc., Princeton, N.J.), chloroquine (Sterling Pharmaceuticals, Sydney, Australia), cycloguanil (ICI Australia, Sydney, Australia) and WR99210 (Jacobus Pharmaceutical Company) using a modified radioisotopic method of Desjardins et al. (6). The K1 and TM93-C1088 lines were maintained in vitro as described earlier. A suspension of infected erythrocytes in RPMI 1640 (for chloroquine) or RPMI 1640 LPLF (for atovaquone, cycloguanil, and WR99210) (containing folic acid [0.01 mg/liter] and para-aminobenzoic acid [0.0005 mg/ml]) supplemented (10% serum) culture medium was added to 96-well microplates which were preseeded with varying concentrations of drugs. The final culture suspension of the chloroquine plates had a hematocrit of 2% and contained 0.5 to 0.6% parasitized erythrocytes (>95% rings). [³H]hypoxanthine (10 μCi containing 0.2 μCi) was added to each plate well at the start of the incubation, and the cultures were harvested 48 h later. The final culture suspension for the atovaquone, cycloguanil, and WR99210 plates had a hematocrit of 1.5% and contained 0.3 to 0.4% parasitized erythrocytes (>95% rings). These cultures were initially incubated for 48 h to allow merozoite reinvasion before [³H]hypoxanthine (10 μCi containing 0.2 μCi) was added to each well. The cells were harvested after a further 24-h incubation. The level of incorporation of [³H]hypoxanthine was used as an index of inhibition of parasite growth. The IC₉₀ and IC₅₀ were determined by estimating the drug concentrations that inhibited parasite isotope incorporation by 50 and 90%, respectively, relative to the drug-free control cultures.

(ii) Parent parasites were suspended in RPMI 1640 complete medium and plated in quadruplicate. [³H]hypoxanthine was added at the beginning of the test, and parasites were harvested 48 h later. Curves were best fitted to the quadruplicate readings using Sigmaplot. The IC₉₀ and IC₅₀ of the compounds for the cloned parasites and the standard errors were also calculated using Sigmaplot.

Amplification and sequencing of the P. falciparum CYT b gene. Parasite DNA was isolated using methods described previously (1). A fragment of the CYT b gene (corresponding to nucleotides 12 to 950 of the gene) was amplified using primers cyt1 (5′ TCT TAT TAA TTT AGT AGT TAA AGC ACA C 3′) and cyt2 (5′ ACA GAA TAA TCT CTA GCA CC 3′). PCR was performed using 2 mM MgCl₂, a 0.2 mM concentration of each deoxynucleoside triphosphate (Promega, Madison, Wis.), 75 ng of each primer, and 1.25 U of AmpliTaq Gold (PE Applied Biosystems). The reaction mixture was initially heated at 93°C for 10 min and then cycled at 93°C for 50 s, 45°C for 50 s, and 70°C for 1 min over 40 cycles. PCR products were purified using the QIAEXII purification kit (QIAGEN, Hilden, Germany) and then sequenced using the ABI Prism Big-Dye Terminator Kit. A second pair of primers, cyt3 (5′ AGC AGT AAT TGG ATG AGG AGG)
3′) and cyt b (5′ ATT TTT AAT GCT GTA TCA TAC CTT 3′) were then used to sequence the amplified products from opposite orientations.

Amino acid comparison of the Qb sites regions in CYT b from various species. The sequences were retrieved from the GenBank or Swiss-Prot database and aligned using Pileup (Wisconsin package, version 8.1.0, March 1996 release). The origins of the sequences were as follows (accession numbers are shown in parentheses): horse (sp-P46663), rhesus (sp-P96771), pig (sp-P29496), sheep (sp-P08011), bovine (sp-P06157), rabbit (sp-P08633), human (sp-P01536), chicken (sp-P18946), Drosofila yakuba (sp-P07704), Saccharomyces cerevisiae (gb-X44042), carchegue (sp-P48785), P. falciparum (gb-M94161), Plasmodium vivax (gb-FA05557), P. yoelii (gb-M29800), P. carini (gb-FA07472), and Toxoplasma gondii (gb-M27627).

Genetic typing for P. falciparum lines. Two polymorphic genes, MSP1 and MSP2, were used as markers for genetic typing of P. falciparum isolates and cloned lines. A fragment from each gene was amplified from parasite lines using the protocols described by Ranford-Cartwright et al. (20). The fragment sizes of PCR products from each parasite line were analyzed.

Molecular modeling. The P. falciparum CYT b model was constructed using the Homology software program of the Insight II molecular modeling system from Molecular Simulations Inc. (MSI). The amino acid sequences of chicken, bovine, and P. falciparum CYT b were aligned using Homology. This alignment was further refined using the predicted structurally conserved regions (SCRs) defined by PROMOTIF (12) of the chicken (1BCC [34]) and bovine (1BES [13]). CYT b crystal structures. Where the template and model did not have the same number of residues, suitable loops with appropriate conformations were assigned from protein structures found in the Brookhaven PDB database.

The model was minimized so that splice points between SCRs and loops were fixed where omega bonds had a distance of 1.34 Å and an angle of 180° ± 1°. The model was then subjected to energy minimization to optimize the energy of the predicted structure to the Cambridge Crystallographic Data Centre. Potential atovaquone binding sites in the P. falciparum CYT b model were searched for on the cytoplasmic face of the protein. The binding predictions from GOLD were processed by the software package LUDI (Ligand Design) from MSI and given a score that was correlated to a theoretical dissociation constant. This constant was based on the number and quality of receptor-ligand hydrogen bonds, and receptor-ligand hydrophobic contact area and was used only as a guide to assess potential binding sites.

Minimization of the energy of the predicted P. falciparum- and chicken- and bovine atovaquone-docked complexes was performed using Discover. Contact residues were defined as those residues that resided within approximately 3.0 Å of atovaquone when bound in the putative site. Stigmatellin was positioned into the P. falciparum model based on a superimposition of the P. falciparum and chicken (stigmatellin-bound) CYT b structures.

Nucleotide sequence accession numbers. The P. falciparum CYT b nucleotide sequences reported in this paper are available in the EMBL, GenBank, and DDBJ databases under the accession numbers AF155925 (TM93-C1088), AF155926 (K1-1D4s [parent]), AF155927 (K1-1D4t [ATV-M1]), and AF121046 to AF212049 (ATV-M2 to ATV-M5).

RESULTS

The P. falciparum isolate TM93-C1088 has a marked reduction in susceptibility to atovaquone and a mutation in its CYT b gene. The P. falciparum TM93-C1088 line was isolated from a Thai patient who experienced recrudescence following atovaquone treatment. The K1 line was also isolated from a Thai patient; however, this patient had not been exposed to atovaquone. When measured in vitro, both lines showed a reduced susceptibility to chloroquine, with IC50 of 244.2 ± 21.93 nM and 169.28 ± 8.74 nM, respectively, and to cycloguanil, with IC50 of 679.38 ± 16.86 nM and 229.11 ± 11.35 nM, respectively, but were sensitive to WR99210 with IC50 of 0.20 ± 0.02 nM and 0.13 ± 0.04 nM, respectively. Their susceptibilities to atovaquone differed markedly. The K1 line was susceptible to low concentrations of atovaquone, with IC50 of 169.28 nM and IC50 of 21.93 nM being below 13.6 nM. The TM93-C1088 line was less susceptible to atovaquone, with mean IC50 of 9,974 and 22,717 nM, respectively. These values were at least 730- and 1,600-fold higher, respectively, than those of the K1 line.

The CYT b gene from TM93-C1088 was sequenced compared to the CYT b sequences of 17 laboratory P. falcipa-
parent line to assume any correlation with the mutations (Table 1). The mutants had the same MSP1 and MSP2 genotypes as the parent line, indicating that the mutants did not result from cross-contamination (data not shown).

Most of the mutations occur in amino acids that are highly conserved in eukaryotic CYT b. Protein sequences of CYT b from 16 different eukaryotic species were aligned and compared (data not shown). Of the six mutations, four occurred in amino acids that are highly conserved in eukaryotic CYT b. Y268, M133, and G280 were conserved across all 16 species, and P275 was conserved among 15 species and replaced by an S in P. carinii, indicating the potential importance of these residues in maintaining CYT b structure and function. Two of the six mutations occurred in residues that are unique to primitive eukaryotes. K272 was seen in Plasmodium and Toxoplasma, whereas V284 was unique to the sequenced Plasmodium spp.

P. falciparum CYT b model. A homology model of P. falciparum CYT b was constructed based on the deduced crystal structures 1BCC and 1BE3. The amino acid sequence alignment and α-helical elements of the three proteins are shown in Fig. 2. The SCRs predicted in the P. falciparum CYT b model were comparable to those reported in the chicken and bovine crystal structures with only minor prediction discrepancies between three- and four-residue helices. A Ramachandran plot revealed that the model was of a high quality, with 87% of the residues being in the most-favored regions (data not shown). Profiles 3D also yielded a high result, with the model scoring 86 out of a possible 169. This was comparable to the chicken crystal structure, which scored 97 out of a possible 173. This score of 97 was used as a theoretical maximum due to the program’s not being able to account for amino acids in membrane bound regions of proteins.

Putative binding site for atovaquone in P. falciparum. Potential binding sites for atovaquone were located on the cytoplasmic face of the P. falciparum CYT b model using GOLD. Most sites predicted by GOLD were given LUDI scores below 650. There was only one site predicted in the model (Fig. 3A) that scored well above this, obtaining a value of 950. This site coincided with a region where ubiquinol has been predicted to bind (13, 31, 34) and is the most likely site where atovaquone binds as judged from the LUDI scores of the predicted sites. Also, the equivalent region in the chicken CYT b structure has been found to support stigmatellin and myxothiazol binding (3). The putative contact residues at this atovaquone-binding site after energy minimization of the atovaquone-CYT b complex in P. falciparum were I119, F123, Y126, M133, V140, I141, L144, I258, P260, F264, F267, Y268, L271, V284, L285, and L288 (Fig. 2 and 3A).

Mutations associated with atovaquone resistance. The mutations Y268S, Y268C, M133I, and V284K (Fig. 3B) in Plasmodium CYT b that were associated with atovaquone resistance occurred at putative contact residues in the CYT b model (Fig. 3A and B). The mutations G280D, K272R, and P275T did not occur at any of the predicted contact residues. However, they were all found in tandem with the mutation at the putative contact site residue M133. Residues Y268, M133, and V284 are all within hydrophobic or H-bond interaction distance (approximately 2 to 3Å) of atovaquone when atovaquone was modeled into the putative binding site.

The mutations I258M, F267I, Y268C, and L271V (Fig. 3B) associated with atovaquone resistance in P. yoelii CYT b also occurred at the putative contact residues in the P. falciparum CYT b model (Fig. 3A). The mutation K272R did not occur at a predicted contact residue but was again found to coexist with the contact residue mutation L271V. The mutation L144F or
L144S (Fig. 3B) found in *P. carinii* and *P. berghei* CYT b, respectively (27, 33), also occurred at a contact residue. T142I was the only lone mutation not directly associated with a contact residue.

Equivalent putative binding site for atovaquone in the chicken and bovine CYT b structures. The equivalent atovaquone binding site in bovine and chicken CYT b (Fig. 3C) with atovaquone modelled into them after energy minimisation revealed a significant difference of contact residues from those predicted for *P. falciparum*. The residues I119, F267, V284, L285 and L288 that form one side of the binding pocket in the *P. falciparum* binding site are represented by A278 and V299 in the chicken and bovine equivalent binding site. These residue differences are bolded in Fig. 2 and compared between Fig. 3A and C.

The atovaquone-binding site may overlap with the stigmatellin and myxothiazol binding site. Since stigmatellin and myxothiazol are well-characterized inhibitors in the CYT b Q site, we were interested in whether the mutations in CYT b that are associated with atovaquone resistance also affect stigmatellin and myxothiazol binding. The parent parasites were less susceptible to stigmatellin and myxothiazol than to atovaquone by 530- and 66-fold, respectively, as judged by the IC50s (Table 1). In the five atovaquone-resistant mutant lines, four had significantly higher IC50s of stigmatellin compared with the parent line, with a 2.7-fold maximum difference. However,
ATV-M5 showed a 664-fold decrease in IC₅₀ compared to the parent. The situation was more complicated for myxothiazol. The ATV-M1, ATV-M3, and TM93-C1088 lines had significantly higher IC₅₀s than the parent line by 2.56-, 1.38-, and 10.58-fold, respectively, while ATV-M2, ATV-M4, and ATV-M5 lines had significantly lower IC₅₀s by 1.35-, 2.7-, and 59-fold, respectively. These results suggest that there may possibly be an overlap between the binding sites of atovaquone, stigmatellin, and myxothiazol in P. falciparum CYT b. This argument was supported by the P. falciparum CYT b model, in which the equivalent stigmatellin-binding site overlapped with part of the atovaquone-binding site (data not shown). The V284K mutation appeared to enhance both stigmatellin and myxothiazol binding to parasite CYT b, while the Y268S and G280D mutations appeared to only enhance myxothiazol binding to CYT b. The M133I mutation in contrast appeared to reduce the binding of both compounds to CYT b.

**Atovaquone is potentially active against other protozoans.** P. carinii (33), T. gondii (29) and Eimeria tenella (11) have already been shown to be susceptible to atovaquone. CYT b amino acid sequences for P. carinii and T. gondii were retrieved from GenBank, and their residues share similar properties with the putative atovaquone contact binding site residues from P. falciparum (data not shown).

**DISCUSSION**

Mutations observed in atovaquone resistant parasite CYT b resulted from drug selection. Atovaquone is a potent and selective mitochondrial inhibitor of P. falciparum and P. yoelii,
and its primary site of action is the CYT bc1 complex (10). Resistance to atovaquone has been associated with sequence changes in CYT b in *P. carinii*, *P. yoelii*, and *P. berghei*. In *P. carinii*, mutations resulting in amino acid changes were found in one of the Qo sites in the CYT b gene of the parasites that were isolated from two of four patients who had failed atovaquone prophylaxis (33). However, the direct correlation between mutations and atovaquone resistance could not be further analyzed due to difficulties in culturing *P. carinii*. The direct evidence came from studies of *P. yoelii*, a rodent malaria species, whereby mutations in CYT b conferred atovaquone resistance. In that study, atovaquone-resistant parasites were derived from suboptimal treatment of *P. yoelii*-infected mice with atovaquone (5 mg/kg of body weight). Single base changes were found in the mitochondrial genome of resistant parasites and were located in the cyt b. These changes resulted in single or double amino acid mutations in the parasite CYT b. Parasites that carried mutations in their CYT b were able to resistant and maintain mitochondrial membrane potential in the presence of atovaquone. In contrast, both these functions in the nonmutant parent parasites were inhibited in the same concentrations of atovaquone (24).

In this work, we derived atovaquone-resistant *P. falciparum* mutants from an atovaquone-sensitive clonal line, K1-1D4, by culturing parasites in medium containing subcurative levels of atovaquone with gradually increased concentrations. A single base mutation was detected in the CYT b gene of the parasite (ATV-M1) that survived the initial 20 nM concentration of atovaquone, resulting in an amino acid change of M133I (in the predicted Qo1 region) and a 25-fold increase in the IC50 of atovaquone compared to its nonmutant parent. When this parasite was again subjected to higher concentrations of atovaquone, extra mutations were selected in the parasite CYT b genes. The resultant amino acid changes K272R, P275T, and G280D were also located close to the predicted Qo1 region. These changes were associated with significant increases in IC50 of 220-, 537-, and 897-fold, respectively, higher than that of the parent. These values were also significantly higher than that of the single M133I mutant. The M133I mutation was also seen in an atovaquone-resistant line of *P. berghei* (27). The two *P. falciparum* mutations, Y268S and K272R, were also seen in the *P. yoelii* atovaquone-resistant mutants with Y268C in *P. yoelii* (24). When parasites were subjected directly to 5 × 10−8 M atovaquone, a double mutation in the parasite CYT b protein sequence was selected: L283I and V284K. The V284K change was probably directly responsible for the 76-fold increase in IC50 because L283 has been observed in atovaquone-sensitive parasites. The V284 mutation has also been reported in an atovaquone-resistant line of *P. berghei* (15). Our sequencing of 17 field isolates that had not been exposed to atovaquone revealed only one synonymous change consistent with the strong evidence that CYT b genes in *Plasmodium* are highly conserved (17). Therefore, it is highly likely that the amino acid changes we saw in the atovaquone-resistant parasites resulted directly from the presence of atovaquone.

**Mutation rate.** The rate at which mutations occurred in the CYT b genes can be estimated from the starting number of parasites, the number of generations, and the number of mutants. As an example, the initial M133I mutation occurred in each of the three atovaquone treated cultures. This mutation could have occurred (i) in the original culture and was partitioned to each of the three cultures or (ii) simultaneously in the three individual cultures. If the mutation occurred before the culture was split, then the mutation must have occurred at least one generation prior to splitting in order for each of the three subcultures to contain at least one mutant parasite. At that time, the flask contained approximately 5 × 106 parasites, so to have at least a 10% chance of the mutation occurring, the overall mutation rate must have been at least 1 in 5 × 108 parasites per generation. If the mutation occurred after splitting, then the mutation must have occurred at least once in each of the three flasks at the time the drug was added. At this stage there were approximately 6 × 109 parasites per flask. Thus, to have at least a 10% chance of seeing at least one parasite per flask, the probability of one mutation occurring per flask was 0.54 (i.e., 1 - 0.14) which would give an overall mutation rate of 6 × 10−8 or approximately 1 in 108 parasites per generation. However, if the mutation rate was high enough to see it after splitting, then the rate would have certainly been high enough for the mutation to have occurred before splitting. Therefore, the simplest explanation to fit these observations is that the M133I mutation occurred prior to splitting the cultures.

These calculations only give a minimum mutation rate. The actual mutation could be much higher, for example similar to the one mutation in 106 parasites observed by Safwat et al. (23). If this is the case, then the mutation would almost certainly have occurred earlier during culture and the observed mutation in this experiment would reflect the first mutation leading to atovaquone resistance which chanced to occur following cloning. Three other mutations that occurred in tandem with M133I (K272R, P275T, and G280D) are most likely to have occurred after the initial M133I mutation because all mutants have the M133I mutation. This is also supported by the modeling results suggesting that K272R, P275T, and G280D are not predicted contact residues and that the combination of M133I and one of the other mutations is required to confer resistance.

In the one-step selection, higher concentrations of atovaquone were used and fewer mutants were obtained. The mutation rate is probably similar, but only the mutants that can survive the concentration were selected. Even at an overall mutation rate as low as one mutation per 5 × 108 parasites per generation, a patient with a parasitemia of 1,000/μl in 5 liters of blood, would have a minimum of 10 mutant parasites. Some of the mutants may confer a strong resistance to atovaquone that would result in a parasite recrudescence.

**Subcurative dose of atovaquone can select mutants that would survive curative treatment.** The *P. falciparum* atovaquone-resistant mutants we report here were selected using subcurative levels of atovaquone. It has been reported that the maximum atovaquone levels in serum after the third dose of Malarone were 5.1 μg/ml or 13.9 μM in children (22) and 13.02 μg/ml or 35 μM in adults (32). The maximum concentration of atovaquone following prophylaxis with Malarone was 5.74 μg/ml or 15 μM (26). Since atovaquone has not been shown to concentrate in the cells, the serum level of atovaquone would be similar to that in the infected red blood cells. The IC50 for one of the mutants, ATV-M4, was 23 μM, which indicates that this parasite has the potential to break through Malarone prophylaxis and even survive the curative treatment regimen of Malarone in vivo, although it was selected at subcurative levels of atovaquone.

**Mutations in CYT b affect drug binding.** In order to understand how these mutations affect atovaquone binding, a model for *P. falciparum* CYT b was constructed based on the deduced crystal structures of chicken and bovine CYT b. A potential binding site for atovaquone in *P. falciparum* CYT b was identified in the model. This putative binding site scored 300 points higher, with a K3 orders of magnitude lower than any of the other potential atovaquone-binding sites. The equivalent site in bovine and chicken CYT b with bound atovaquone had a
lower affinity compared with the site in P. falciparum. Residues I119, F267, V284, L285, and L288 in the P. falciparum binding site can therefore support tighter binding of atovaquone than A278 and V299 in the equivalent positions in the chicken and bovine binding sites. This is consistent with Fry and Pudney’s result that atovaquone was 2,000-fold more active against P. falciparum mitochondria function than in rat liver (10).

In the P. falciparum model, three of the six P. falciparum mutations, four of the five P. yoelii mutations, and one of the P. carinii mutations, and all three of the P. berghei mutations were predicted to cause a decrease in binding affinity for atovaquone in the putative binding site. The P. falciparum Y268S and P. yoelii Y268C mutations resulted in a dramatically reduced hydrophobic contact with atovaquone as well as altering H-bond formation potential. This could explain the marked reduction in susceptibility to atovaquone by 9,354-fold and 1,667-fold, respectively. The V284K mutation added a steric and charge alteration into the hydrophobic end of the binding pocket. This was consistent with a 76-fold change in susceptibility to atovaquone.

In contrast, the M133I substitution resulted in a steric alteration at the binding site consistent with a 25-fold reduction in susceptibility to atovaquone. The mutations K272R, P275T, and the discovery of other mutations or the elucidation of an overlap of the phenyalanine ring with atovaquone. The L144F mutation in P. carinii and the T142I mutation in P. falciparum predicted to cause a decrease in binding affinity for atovaquone through F123. Given that the amino acid sequence of the T142I mutation in P. falciparum was identical to P. carinii at this site, the two mutations seen in P. carinii may potentially occur in P. falciparum with a similar effect. The current data for atovaquone resistance supports the location of this putative binding site and the discovery of other mutations or the elucidation of an atovaquone-CYT bc complex crystal model will provide a more detailed analysis.

Atovaquone is the main component of the new antimalarial drug, Malaron, which is now available in many countries for the treatment of malaria. Atovaquone is also being used for the treatment of Plasmodium, mainly in human immunodeficiency virus and AIDS patients. This information on the atovaquone-binding site may provide a guide for exploring the use of atovaquone in treating other opportunistic parasites. In view of its increasing use, it is important to understand the mechanism of resistance and to monitor the emergence of parasites resistant to this drug. The discovery of mutations associated with atovaquone resistance provides a molecular marker for monitoring the development and spread of drug-resistant parasites in the field.

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**REFERENCES**


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