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# 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 $bc_1$ (CYT $bc_1$) 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 $10^5$ 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-dioxobenzoxythiazol (UHDBT), which are structurally similar to atovaquone, have been shown to bind at the ubihydroquinone ($Q_o$) site of CYT b and inhibit electron transport. Single point mutations within the $Q_o$ site confer resistance to these inhibitors in a variety of microorganisms (7). Two mutations in close proximity to the $Q_o$ site in *Pneumocystis carinii* are associated with atovaquone prophylaxis failure (33). 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 $Q_o$ 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* genes 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 $bc_1$ 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

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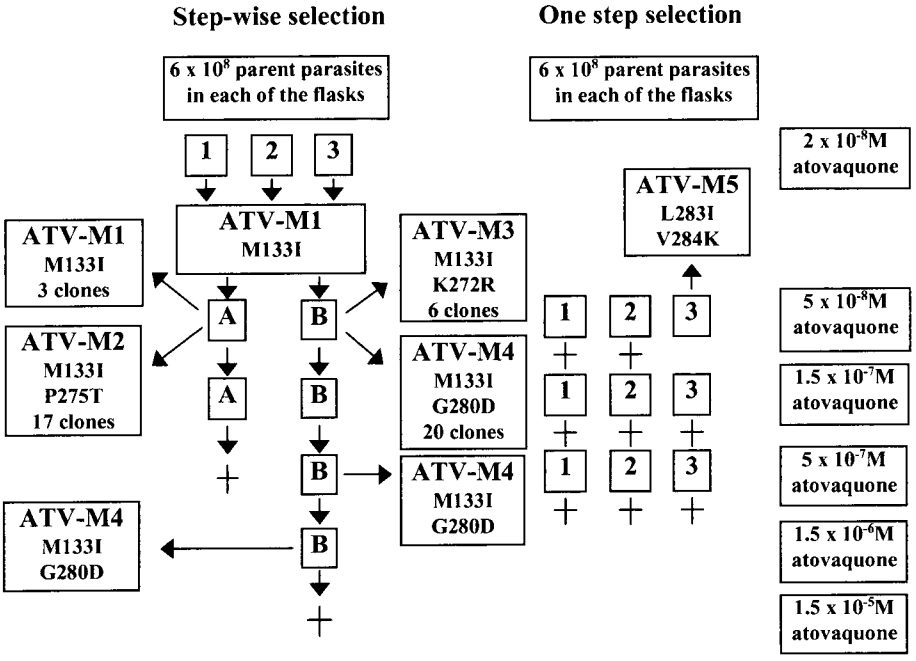


FIG. 1. Illustration of the procedures involved in the in vitro selection of atovaquone-resistant mutants.

laboratory. The origins and sources of HB3, 3D7, T9-94, MCK, D10, Palo Alto, Itg2, 7G8, W/L, NF36, MS2, AE7, FCQ50, and FCQ79 have been described elsewhere (15). Dd2 has been described by Oduola et al. (19).

**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 $2 \times 10^{-8}$ M atovaquone, a concentration equivalent to the 90% inhibitory concentration ($IC_{90}$) ($2.2 \times 10^{-8}$ 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 $5 \times 10^{-8}$, $1.5 \times 10^{-7}$, $5 \times 10^{-7}$, $1.5 \times 10^{-6}$, and $1.5 \times 10^{-5}$ 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 treatment when parasitemias reached approximately 3%. Three sets of three flasks of parasites were cultured in the presence of $5 \times 10^{-8}$, $1.5 \times 10^{-7}$, and $5 \times 10^{-7}$ M atovaquone. The three sets of cultures were maintained at these concentrations for 6 weeks. The surviving parasites were cryopreserved and extracted for DNA.

**Cloning of atovaquone-resistant mutant K1-1D4 parasites.** Parasites that survived $2 \times 10^{-8}$ M atovaquone initially and $5 \times 10^{-8}$ 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-amicobenzoic acid [0.0005 mg/liter]) supplemented (10% serum) culture medium was added to 96-well microplates which were predosed 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). [$^3$H]hypoxanthine (10 μl 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 [$^3$H]hypoxanthine (10 μl containing 0.2 μCi) was added to each well. The cells were harvested after a further 24-h incubation. The level of incorporation of [$^3$H]hypoxanthine was used as an index of inhibition of parasite growth. The $IC_{50}$s and $IC_{90}$s 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) The parent clone and the selected mutant clones of K1-1D4 were tested for their susceptibilities to atovaquone, stigmatellin (Fluka Chemika-Biochemika, Buchs, Switzerland), and myxothiazol (ICN, Costa Mesa, Calif.). In this experiment, parasites were suspended in RPMI 1640 complete medium and plated in quadruplicate. [$^3$H]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 $IC_{50}$s and $IC_{90}$s 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 cytb1 (5′ CTC TAT TAA TTT AGT TAA AGC ACA C 3′) and cytb2 (5′ ACA GAA TAA TCT CTA GCA CC 3′). PCR was performed using 2 mM $Mg^{2+}$, a 0.2 mM concentration of each deoxynucleoside triphosphate (Promega, Madison, Wis.), 75 ng of each primer, and 1.25 U of AmpliTaqGold (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, cytb3 (5′ AGC AGT AAT TTG GAT ATG TGG AGG

3′) and cytb4 (5′ ATT TTT AAT GCT GTA TCA TAC CCT 3′), were then used to sequence the amplified products from opposite orientations.

**Amino acid comparison of the $Q_o$ site 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-P48665), rhinoceros (sp-Q96071), pig (sp-P24964), sheep (sp-P24959), bovine (sp-P00157), rabbit (sp-P34863), human (sp-P00156), chicken (sp-P18946), *Drosophila yakuba* (sp-P07704), *Saccharomyces cerevisiae* (gb-X84042), carrageen (sp-P48875), *P. falciparum* (gb-M99416), *Plasmodium vivax* (gb-AF055587), *P. yoelii* (gb-M29000), *P. carinii* (gb-AF074872), and *Toxoplasma gondii* (gb-AF015627).

**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 (1BE3 [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 refined 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 MSI program Discover was then used to minimize the energy of the structure to an RMS deviation of less than $10^{-6}$ using steepest descents and conjugate gradients taking into account morse and cross terms and charges. Amino acid substitutions were made using Homology. The *P. falciparum* CYT *b* model was validated using Profiles 3D (within the Homology program) and Procheck (18).

Modeling of the antimalarial drug atovaquone into potential binding sites in the chicken, bovine, and *P. falciparum* CYT *b* structures was performed using the program for genetic optimization for ligand docking (GOLD) (14) from 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 the receptor-ligand hydrophobic contact area and was only used 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 DDJB databases under the accession numbers AF155925 (TM93-C1088), AF155926 (K1-1D4s [parent]), AF155927 (K1-1D4r [ATV-M1]), and AF212046 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 $IC_{50}$s of 244.23 ± 21.93 nM and 169.28 ± 8.74 nM, respectively, and to cycloguanil, with $IC_{50}$s of 679.38 ± 16.86 nM and 229.11 ± 113.38 nM, respectively, but were sensitive to WR99210, with $IC_{50}$s 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 the $IC_{50}$s and $IC_{90}$s being below 13.6 nM. The TM93-C1088 line was less susceptible to atovaquone, with mean $IC_{50}$s and $IC_{90}$s 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 and compared to the CYT *b* sequences of 17 laboratory *P. falciparum* lines, including K1. These 17 lines had not been exposed to atovaquone. Based on the PCR-amplified polymorphic markers, MSP1 and MSP2, each parasite line had a unique genotype (data not shown). Sequencing of a 939-bp cyt *b* fragment (85% of the gene) from these 17 laboratory lines revealed a high degree of conservation despite differences in the lines origins and other marker genes. The only synonymous nucleotide substitution found in the 17 lines of CYT *b* genes was in 7G8. Sequencing of the CYT *b* gene from TM93-C1088 revealed a unique single base change, A to C at nucleotide 803 (sequence numbers starting from the start codon ATG). This resulted in an amino acid change from $Y_{(TAT)}268$ to $S_{(TCT)}268$.

**In vitro development of atovaquone resistance in the *P. falciparum* K1 cloned line is also associated with mutations in their CYT *b* genes.** The ATV-M1 parasites were selected after continuous exposure of the cloned parent parasites to $2 \times 10^{-8}$ M atovaquone. A single base mutation was found in the CYT *b* gene of the ATV-M1 parasites from each of the three cultures. The mutation G to A at nucleotide 399 resulted in an amino acid change from $M_{(ATG)}133$ to $I_{(ATA)}133$.

The ATV-M1 parasites were then cultured in two flasks containing $5 \times 10^{-8}$ M atovaquone. Sequencing of the CYT *b* gene from parasites that survived this concentration of atovaquone revealed an M133I mutation with mixed signals at several other positions, indicating multiple parasite populations. The parasites were then cloned using a limiting-dilution method to yield a total of 20 individual clones from flask A and 26 clones from flask B. CYT *b* genes were amplified and sequenced from these cloned parasites. In flask A, 3 clones had the single mutation of M133I (ATV-M1) and 17 had the double mutation M133I and $P_{(CCA)}275T_{(ACA)}$(ATV-M2). In flask B, 6 clones had the double mutation M133I and $K_{(AAA)}272R_{(AGA)}$(ATV-M3) and 20 had the double mutation M133I and $G_{(GGT)}280D_{(GAT)}$(ATV-M4).

ATV-M2 and ATV-M3 tolerated $1.5 \times 10^{-7}$ M atovaquone but failed to grow when the atovaquone concentration was increased to $5 \times 10^{-7}$ M. ATV-M4 parasites grew well in $1.5 \times 10^{-6}$ M atovaquone but failed to grow in $1.5 \times 10^{-5}$ M atovaquone. Figure 1 illustrates the procedures and results.

ATV-M5 was selected from one of the three cultures in which the parent parasites were subjected to $5 \times 10^{-8}$ M atovaquone. The cyt *b* sequence of this line had three adjacent mutations that resulted in changes of two adjacent amino acids: $L_{(ATT)}283I_{(ATA)}$ and $V_{(GTA)}284K_{(AAA)}$. None of the three cultures that were subjected to $1.5 \times 10^{-7}$ or $5 \times 10^{-7}$ M atovaquone grew resistant mutants.

The susceptibilities to atovaquone of the mutant parasite clones ATV-M1 to ATV-M5 were measured and compared to those of the parent clone and the TM93-C1088 isolate (Table 1). All mutant parasites showed a significant increase in $IC_{50}$s (Table 1) and $IC_{90}$s (data not shown) of atovaquone. The TM93-C1088 isolate (Y268S) showed a 9,354-fold-higher $IC_{50}$ than the K1 parent line. Among the K1 mutants, ATV-M4 (M133I-G280D), which survived the highest test concentration of atovaquone (1,500 nM) in culture, had an 897-fold increase in its $IC_{50}$. This was followed by ATV-M3 (M133I-K272R), with a 537-fold increase; ATV-M2 (M133I-P275T), with a 220-fold increase; and ATV-M1 (M133I alone), with a 25-fold increase (Table 1). ATV-M5 (L283I-V284K) had a 76-fold increase in $IC_{50}$ (Table 1). The $IC_{50}$s of the mutants correlate well with the concentration of atovaquone at which they were originally selected. The mutations have been stable ever since the mutant parasites were cultured in drug-free medium for 3 months.

By contrast, the susceptibilities of the K1 mutants to chloroquine were not markedly different enough from that of the

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

TABLE 1. Susceptibilities of parasites to various drugs

| Parasite | Atovaquone concn in culture | Mutation(s) in CYT *b* | Atovaquone | | Stigmatellin | | Myxothiazole | | Chloroquone | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | $IC_{50}$ ± SE (nM) | Index | $IC_{50}$ ± SE (nM) | Index | $IC_{50}$ ± SE (nM) | Index | $IC_{50}$ ± SE (nM) | Index |
| Parent | 0 | None | 3.13 ± 0.61 | 1 | 1,661.61 ± 109.43 | 1 | 208.38 ± 10.63 | 1 | 153.09 ± 0.71 | 1 |
| ATV-M1 | 20 | M133I | 78.62 ± 2.74[a] | 25.12 | 4,513.67 ± 374.32[a] | 2.72 | 534.38 ± 40.07[a] | 2.56 | 188.12 ± 8.68[a] | 1.12 |
| ATV-M2 | 150 | M1331, P275T | 689.48 ± 52.89[a] | 220.28 | 2,954.81 ± 88.38[a] | 1.78 | 153.41 ± 6.16[a] | 0.74 | 176.78 ± 5.71[a] | 1.12 |
| ATV-M3 | 150 | M1331, K272R | 1,680.83 ± 211.18[a] | 537.01 | 2,087.04 ± 64.41[b] | 1.26 | 288.97 ± 15.37[a] | 1.39 | 183.42 ± 11.32[a] | 1.18 |
| ATV-M4 | 1,500 | M1331, G280D | 2,808.82 ± 360.92[a] | 897.39 | 2,175.36 ± 56.83[a] | 1.31 | 76.36 ± 2.05[a] | 0.37 | 184.69 ± 9.81[a] | 1.16 |
| ATV-M5 | 150 | L2831, V284K | 240.17 ± 33.12[a] | 76.73 | 2.56 ± 0.31[a] | 0.0015 | 3.47 ± 0.64[a] | 0.17 | 199.73 ± 6.92[a] | 1.39 |
| TM93-C1088 | ND[c] | Y268S | 29,276.78 ± 2457.11[a] | 9,353.60 | 1,635.05 ± 77.85 | 0.98 | 2,202.15 ± 66.92[a] | 10.56 | 329.18 ± 17.19[a] | 2.10 |

[a] Significantly different from the parent ($P < 0.0001$).
[b] Significantly different from the parent ($P < 0.005$).
[c] ND, not determined.

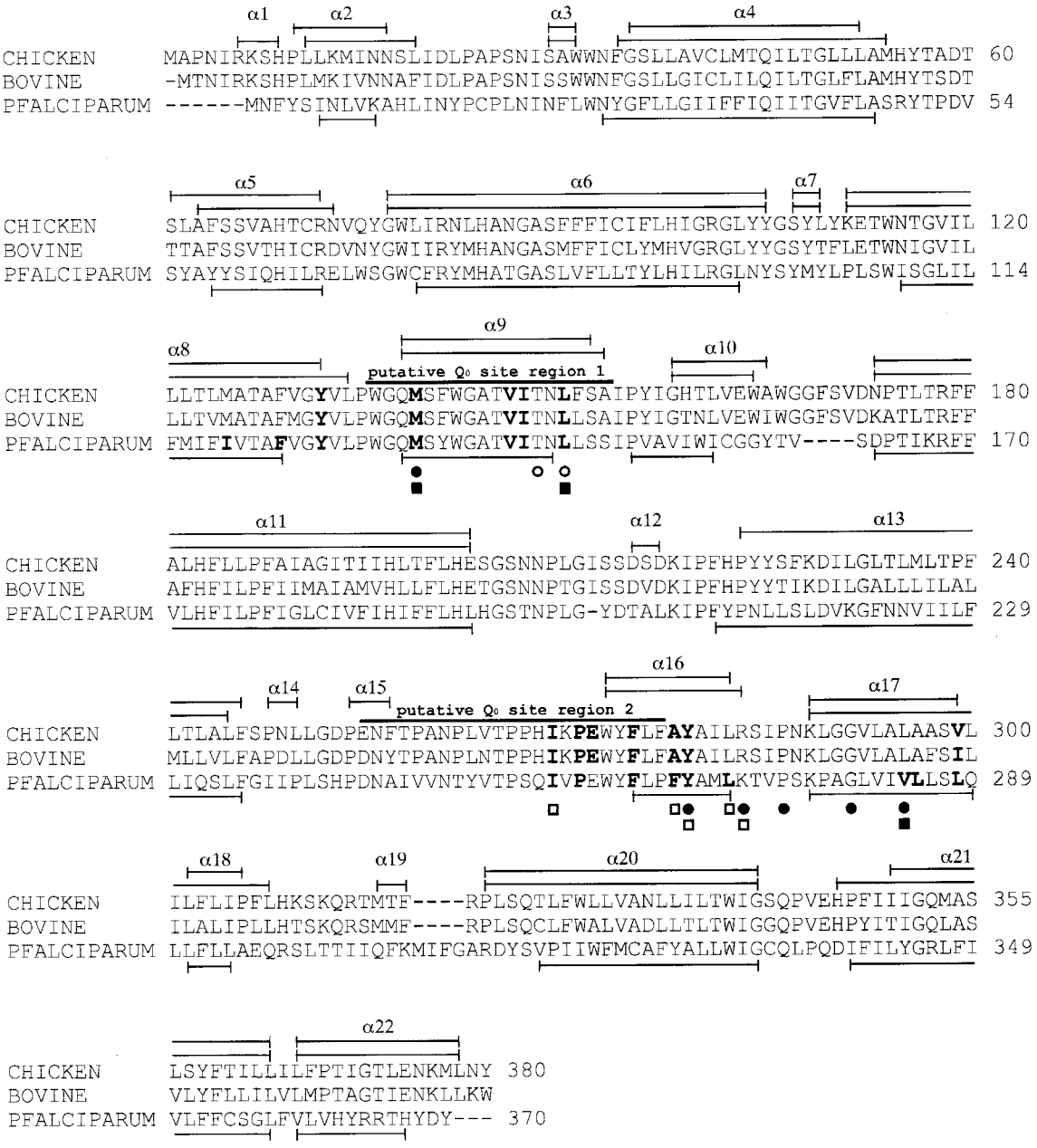


FIG. 2. Amino acid sequence alignment of chicken, bovine, and *P. falciparum* CYT *b* with their SCR elements represented by bars on the top, second top, and bottom, respectively. Putative residues involved in atovaquone binding are shown in boldface type. Residues associated with atovaquone resistance are marked as: *P. falciparum* (●), *P. yoelii* (□), *P. carinii* (○), and *P. berghei* (■). The putative $Q_o$ sites are labeled.

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* $Q_o$ 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 $IC_{50}$s (Table 1). In the five atovaquone-resistant mutant lines, four had significantly higher $IC_{50}$s of stigmatellin compared with the parent line, with a 2.7-fold maximum difference. However,

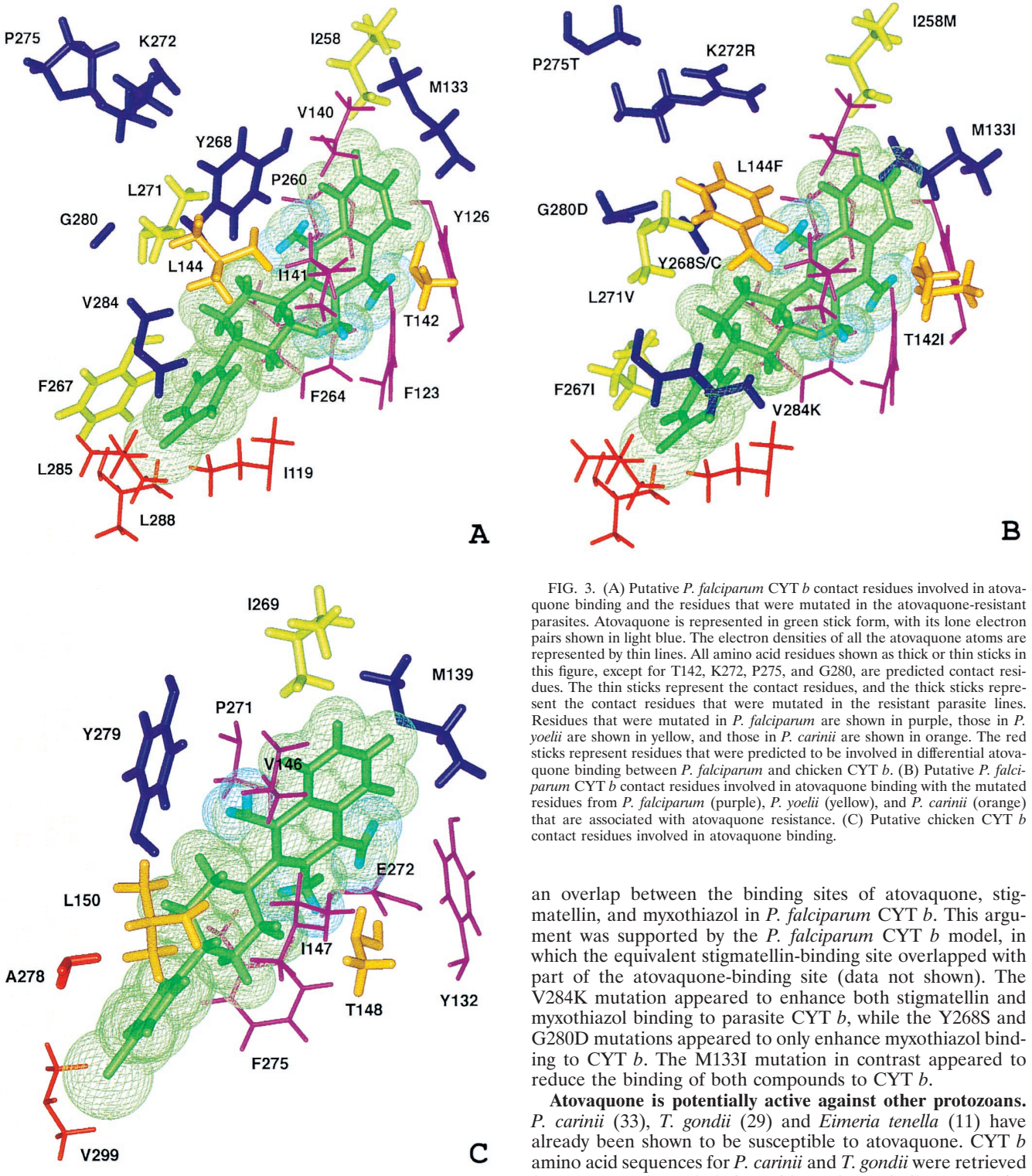


FIG. 3. (A) Putative *P. falciparum* CYT *b* contact residues involved in atovaquone binding and the residues that were mutated in the atovaquone-resistant parasites. Atovaquone is represented in green stick form, with its lone electron pairs shown in light blue. The electron densities of all the atovaquone atoms are represented by thin lines. All amino acid residues shown as thick or thin sticks in this figure, except for T142, K272, P275, and G280, are predicted contact residues. The thin sticks represent the contact residues, and the thick sticks represent the contact residues that were mutated in the resistant parasite lines. Residues that were mutated in *P. falciparum* are shown in purple, those in *P. yoelii* are shown in yellow, and those in *P. carinii* are shown in orange. The red sticks represent residues that were predicted to be involved in differential atovaquone binding between *P. falciparum* and chicken CYT *b*. (B) Putative *P. falciparum* CYT *b* contact residues involved in atovaquone binding with the mutated residues from *P. falciparum* (purple), *P. yoelii* (yellow), and *P. carinii* (orange) that are associated with atovaquone resistance. (C) Putative chicken CYT *b* contact residues involved in atovaquone binding.

ATV-M5 showed a 664-fold decrease in $IC_{50}$ compared to the parent. The situation was more complicated for myxothiazol. The ATV-M1, ATV-M3, and TM93-C1088 lines had significantly higher $IC_{50}$s 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 $IC_{50}$s 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 $bc_1$ 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 $Q_o$ site regions 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 respirate 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 cloned 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 $Q_o$I region) and a 25-fold increase in the $IC_{50}$ 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 $Q_o$ region. These changes were associated with significant increases in $IC_{50}$s 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 $5 \times 10^{-8}$ 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 $IC_{50}$, because I283 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 $5 \times 10^7$ parasites, so to have at least a 10% chance of the mutation occurring, the overall mutation rate must have been at least 1 in $5 \times 10^8$ 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 $6 \times 10^8$ 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., $1 - 0.1^3$) which would give an overall mutation rate of $6 \times 10^8/0.54$ or approximately 1 in $10^8$ 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 $10^5$ 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 at one mutation per $5 \times 10^8$ 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 $IC_{90}$ 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 $K_i$ 3 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, one of the two *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. yoelli* 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 of 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 G280D that occurred only in tandem with M133I most likely caused structural alterations coupled with the steric hindrance through the M133I change. The structural alterations may have enhanced the alteration by M133I but may have not caused enough change to confer resistance themselves.

The L144F mutation in *P. carinii* and *P. berghei* was predicted to cause a steric hindrance into the binding site due to the overlap of the phenyalanine ring with atovaquone. The T142I mutation in *P. carinii* can only be predicted to have caused an indirect structural disruption to the binding site through F123. Given that the amino acid sequence of *P. falciparum* was almost 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 $bc_1$ crystal complex will provide a more detailed analysis.

Atovaquone is the main component of the new antimalarial drug, Malarone, which is now available in many countries for the treatment of malaria. Atovaquone is also being used for the treatment *Pneumocystis*, 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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