Toxoplasmosis caused by the protozoan parasite *Toxoplasma gondii* is a widespread disease affecting primarily immunocompromised and pregnant individuals [1]. Atovaquone is a recently introduced anti-malarial compound with broad spectrum activity against various apicomplexan parasites [2–5] including *T. gondii* [6]. Approved by the FDA in 1995, this drug is a potent and specific inhibitor of the cytochrome *b*-c1 complex [7], an essential respiratory enzyme present in the inner mitochondrial membrane. Two recent studies on treatment of malaria during pregnancy show that atovaquone is a remarkably well tolerated molecule, safe for both the mother and the fetus [8,9]. Unfortunately, there is strong evidence that the targeted parasites are able to spontaneously develop drug resistance by mutation of amino acid residues located in or near the atovaquone-binding site on cytochrome *b* [13]. Because the yeast *b*-c1 complex is also strongly inhibited by atovaquone, we have previously developed *Saccharomyces cerevisiae* as a model to study cytochrome *b* mutations conferring atovaquone resistance in *Pneumocystis* [10,11] and *Plasmodium* species [12]. In the present study, we have successfully transferred two mutations associated with atovaquone resistance in *T. gondii* into the yeast *b*-c1 complex by the biolistic method [11].

Growth of the two mutated strains was monitored in a non-fermentable medium in order to study the impact of the mutations on the yeast cells’ respiration. The growth curves revealed that the wild-type strain and both the M139L and I269L mutant strains displayed a doubling time of 6 (±0.5) h at 30 °C (data not shown). This result suggests that, contrary to what was observed previously with some mutations associated with atovaquone resistance in *Plasmodium* [12], the two cytochrome *b* mutations associated with atovaquone resistance in *T. gondii* do not significantly alter the yeast’s respiration. The bulk of the molecular interactions between atovaquone and cytochrome *b* are essentially hydrophobic with a network of aromatic and aliphatic side chains surrounding the inhibitor.

Toxoplasmosis is caused by the protozoan parasite *Toxoplasma gondii* and is a widespread disease affecting primarily immunocompromised and pregnant individuals [1]. Atovaquone is a recently introduced anti-malarial compound with broad spectrum activity against various apicomplexan parasites [2–5] including *T. gondii* [6]. Approved by the FDA in 1995, this drug is a potent and specific inhibitor of the cytochrome *b*-c1 complex [7], an essential respiratory enzyme present in the inner mitochondrial membrane. Two recent studies on treatment of malaria during pregnancy show that atovaquone is a remarkably well tolerated molecule, safe for both the mother and the fetus [8,9]. Unfortunately, there is strong evidence that the targeted parasites are able to spontaneously develop drug resistance by mutation of amino acid residues located in or near the atovaquone-binding site on cytochrome *b*. Because the yeast *b*-c1 complex is also strongly inhibited by atovaquone, we have previously developed *Saccharomyces cerevisiae* as a model to study cytochrome *b* mutations conferring atovaquone resistance in *Pneumocystis* [10,11] and *Plasmodium* species [12]. In the present study, we have successfully transferred two mutations associated with atovaquone resistance in *T. gondii* into the yeast *b*-c1 complex [13]. Because the yeast *b*-c1 complex is also strongly inhibited by atovaquone, we have previously developed *Saccharomyces cerevisiae* as a model to study cytochrome *b* mutations conferring atovaquone resistance in *Pneumocystis* [10,11] and *Plasmodium* species [12]. In the present study, we have successfully transferred two mutations associated with atovaquone resistance in *T. gondii* into the yeast *b*-c1 complex by the biolistic method [11].

Growth of the two mutated strains was monitored in a non-fermentable medium in order to study the impact of the mutations on the yeast cells’ respiration. The growth curves revealed that the wild-type strain and both the M139L and I269L mutant strains displayed a doubling time of 6 (±0.5) h at 30 °C (data not shown). This result suggests that, contrary to what was observed previously with some mutations associated with atovaquone resistance in *Plasmodium* [12], the two cytochrome *b* mutations associated with atovaquone resistance in *T. gondii* do not significantly alter the yeast’s respiration. The bulk of the molecular interactions between atovaquone and cytochrome *b* are essentially hydrophobic with a network of aromatic and aliphatic side chains surrounding the inhibitor.

Toxoplasmosis caused by the protozoan parasite *Toxoplasma gondii* is a widespread disease affecting primarily immunocompromised and pregnant individuals [1]. Atovaquone is a recently introduced anti-malarial compound with broad spectrum activity against various apicomplexan parasites [2–5] including *T. gondii* [6]. Approved by the FDA in 1995, this drug is a potent and specific inhibitor of the cytochrome *b*-c1 complex [7], an essential respiratory enzyme present in the inner mitochondrial membrane. Two recent studies on treatment of malaria during pregnancy show that atovaquone is a remarkably well tolerated molecule, safe for both the mother and the fetus [8,9]. Unfortunately, there is strong evidence that the targeted parasites are able to spontaneously develop drug resistance by mutation of amino acid residues located in or near the atovaquone-binding site on cytochrome *b*. Because the yeast *b*-c1 complex is also strongly inhibited by atovaquone, we have previously developed *Saccharomyces cerevisiae* as a model to study cytochrome *b* mutations conferring atovaquone resistance in *Pneumocystis* [10,11] and *Plasmodium* species [12]. In the present study, we have successfully transferred two mutations associated with atovaquone resistance in *T. gondii* into the yeast *b*-c1 complex by the biolistic method [11].

Growth of the two mutated strains was monitored in a non-fermentable medium in order to study the impact of the mutations on the yeast cells’ respiration. The growth curves revealed that the wild-type strain and both the M139L and I269L mutant strains displayed a doubling time of 6 (±0.5) h at 30 °C (data not shown). This result suggests that, contrary to what was observed previously with some mutations associated with atovaquone resistance in *Plasmodium* [12], the two cytochrome *b* mutations associated with atovaquone resistance in *T. gondii* do not significantly alter the yeast’s respiration. The bulk of the molecular interactions between atovaquone and cytochrome *b* are essentially hydrophobic with a network of aromatic and aliphatic side chains surrounding the inhibitor.

Toxoplasmosis caused by the protozoan parasite *Toxoplasma gondii* is a widespread disease affecting primarily immunocompromised and pregnant individuals [1]. Atovaquone is a recently introduced anti-malarial compound with broad spectrum activity against various apicomplexan parasites [2–5] including *T. gondii* [6]. Approved by the FDA in 1995, this drug is a potent and specific inhibitor of the cytochrome *b*-c1 complex [7], an essential respiratory enzyme present in the inner mitochondrial membrane. Two recent studies on treatment of malaria during pregnancy show that atovaquone is a remarkably well tolerated molecule, safe for both the mother and the fetus [8,9]. Unfortunately, there is strong evidence that the targeted parasites are able to spontaneously develop drug resistance by mutation of amino acid residues located in or near the atovaquone-binding site on cytochrome *b*. Because the yeast *b*-c1 complex is also strongly inhibited by atovaquone, we have previously developed *Saccharomyces cerevisiae* as a model to study cytochrome *b* mutations conferring atovaquone resistance in *Pneumocystis* [10,11] and *Plasmodium* species [12]. In the present study, we have successfully transferred two mutations associated with atovaquone resistance in *T. gondii* into the yeast *b*-c1 complex by the biolistic method [11].

Growth of the two mutated strains was monitored in a non-fermentable medium in order to study the impact of the mutations on the yeast cells’ respiration. The growth curves revealed that the wild-type strain and both the M139L and I269L mutant strains displayed a doubling time of 6 (±0.5) h at 30 °C (data not shown). This result suggests that, contrary to what was observed previously with some mutations associated with atovaquone resistance in *Plasmodium* [12], the two cytochrome *b* mutations associated with atovaquone resistance in *T. gondii* do not significantly alter the yeast’s respiration. The bulk of the molecular interactions between atovaquone and cytochrome *b* are essentially hydrophobic with a network of aromatic and aliphatic side chains surrounding the inhibitor.

Toxoplasmosis caused by the protozoan parasite *Toxoplasma gondii* is a widespread disease affecting primarily immunocompromised and pregnant individuals [1]. Atovaquone is a recently introduced anti-malarial compound with broad spectrum activity against various apicomplexan parasites [2–5] including *T. gondii* [6]. Approved by the FDA in 1995, this drug is a potent and specific inhibitor of the cytochrome *b*-c1 complex [7], an essential respiratory enzyme present in the inner mitochondrial membrane. Two recent studies on treatment of malaria during pregnancy show that atovaquone is a remarkably well tolerated molecule, safe for both the mother and the fetus [8,9]. Unfortunately, there is strong evidence that the targeted parasites are able to spontaneously develop drug resistance by mutation of amino acid residues located in or near the atovaquone-binding site on cytochrome *b*. Because the yeast *b*-c1 complex is also strongly inhibited by atovaquone, we have previously developed *Saccharomyces cerevisiae* as a model to study cytochrome *b* mutations conferring atovaquone resistance in *Pneumocystis* [10,11] and *Plasmodium* species [12]. In the present study, we have successfully transferred two mutations associated with atovaquone resistance in *T. gondii* into the yeast *b*-c1 complex by the biolistic method [11].

Growth of the two mutated strains was monitored in a non-fermentable medium in order to study the impact of the mutations on the yeast cells’ respiration. The growth curves revealed that the wild-type strain and both the M139L and I269L mutant strains displayed a doubling time of 6 (±0.5) h at 30 °C (data not shown). This result suggests that, contrary to what was observed previously with some mutations associated with atovaquone resistance in *Plasmodium* [12], the two cytochrome *b* mutations associated with atovaquone resistance in *T. gondii* do not significantly alter the yeast’s respiration. The bulk of the molecular interactions between atovaquone and cytochrome *b* are essentially hydrophobic with a network of aromatic and aliphatic side chains surrounding the inhibitor.
not severely impair the ubiquinol binding properties of the yeast $bc_1$ complex.

Ubiquinol–cytochrome $c$ reductase activities of the purified $bc_1$ complexes from the wild-type and mutated yeast strains were measured in the presence of increasing concentrations of atovaquone to test whether sensitivity of the enzymes to the drug was altered by the cytochrome $b$ mutations. We have shown previously that the $bc_1$ complex from wild-type yeast is sensitive to atovaquone with an inhibitor concentration of approximately 25 nM required for 50% inhibition of the activity of the pure enzyme [7]. Both cytochrome $b$ mutations that confer atovaquone resistances to $T. gondii$ also alter the IC$_{50}$ for inhibition of the yeast $bc_1$ complex and by different amounts (Fig. 2A). The I269L mutation has the smallest effect with approximately a two-fold increase in resistance, with an IC$_{50}$ value for the pure $bc_1$ complex of 60 nM. The M139L mutation results in a higher level of resistance to the atovaquone, with an IC$_{50}$ value of 400 nM, which corresponds to a 16-fold
increase over the concentration required to inhibit the wild-type enzyme.

The two T. gondii cytochrome b mutations showing atovaquone resistance were built into the previously modeled atovaquone-bound center P site [7], and the structural changes resulting from the mutations were modeled in silico (Fig. 1B and C). Both residues are located in a hydrophobic region of the binding pocket and interact directly with the phenyl moiety of the drug’s naphthoquinone ring system.

Modeling of the M139L mutation revealed the molecular influence of the Met-139 in atovaquone binding (Fig. 1B). Phenyl ring systems like the naphthoquinone group are well known to interact with the sulfur-containing side chain of methionine through polarization of the C–H bonds [15].

Modeling of the I269L mutation shows a slight modification in size of the aliphatic side chain. In the structure of the wild-type enzyme, the Ile-269 clearly optimizes both van der Waals and electrostatic interactions with the atovaquone. On the contrary, the mutation into Leu-269 decreases the volume available in this region of the hydrophobic pocket, which predictably interferes with atovaquone binding by pushing the inhibitor slightly out of the binding site (Fig. 1C).

The energy required for binding atovaquone was calculated from the modeled structures using a fixed subset of amino acids surrounding the inhibitor. The calculated changes in binding energy were then compared to the experimentally measured changes in IC50 values. The changes in atovaquone binding energy with the bci complexes from the mutants relative to the binding energy obtained for the wild-type enzyme and the changes in IC50 values for the mutant enzymes compared with the wild-type enzyme are shown in Fig. 2B. The relative increase in calculated binding energy correlates well with the relative increase in IC50 values for the bci complexes with both mutations.

In a previous study of mutations conferring atovaquone resistance in pathogenic Pneumocystis fungi, we showed that half of the mutations were distal from the drug binding pocket and these were able to remotely modify the drug binding properties of the bci complex [11]. More recently, we established that mutations that confer atovaquone resistance in Plasmodium affect residues located on or near the EF helix and interact directly with the atovaquone [12]. Interestingly, both T. gondii mutated residues that we have duplicated in yeast in the present study are located in the binding pocket and interact directly with the naphthoquinone ring system of the atovaquone.

Molecular modeling was able to account for the effects of both point mutations. In the case of M139L, the mutation removes a sulfur-containing residue localized in line to the polarized hydrogen atoms of the large aromatic ring system of the atovaquone and suppresses a significant interaction between the drug and the binding pocket. As the hydrogen atoms of the naphthoquinone group are slightly electropositive, they are likely to interact with the large electronegative sulfur atom of Met-139. Because the M139L mutation replaces the sulfur–aromatic link by a weaker hydrophobic interaction, the affinity of the atovaquone for the binding pocket is decreased.

The molecular modeling also revealed that the I269L mutation, by inserting a bulkier residue, is a typical example of resistance acquired by increasing steric constraints on the inhibitor. In Plasmodium, the previously studied I269M mutation caused a similar effect but with a more dramatic increase in the resistance level [12]. In addition, several other atovaquone resistant mutations (I147V, L150F and L275F) found in Pneumocystis have been shown to have analogous steric effects in the binding pocket [11].

Previous computer calculations applied on six mutations that confer atovaquone resistance in Pneumocystis [11] and five that confer resistance in Plasmodium [12] were successful in modeling the effect of those mutations on the atovaquone binding energy. With the two current mutations from T. gondii, we now have a database of 13 point mutations surrounding the atovaquone binding site. The IC50 values for inhibition by atovaquone and theoretically calculated changes in binding energies for these mutations are a useful tool to predict and explain how additional atovaquone resistant mutations might arise.

Acknowledgement

This research was supported by National Institutes of Health Research Grant GM 20379.

References


