

Short communication

## Molecular basis of *Toxoplasma gondii* atovaquone resistance modeled in *Saccharomyces cerevisiae*

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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 *bc*<sub>1</sub> 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 *bc*<sub>1</sub> 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* [13] into the yeast cytochrome *b* gene. This has allowed us to biochemically confirm the linkage of atovaquone resistance to the cytochrome *b* mutations and to predict at the molecular level the mechanism by which *T. gondii* may counter the efficacy of this potential anti-toxoplasma drug.

The molecular target of atovaquone is now known to be the ubiquinol oxidation pocket at center P of the cytochrome

*bc*<sub>1</sub> complex [7]. This binding pattern of atovaquone is similar to the one recently found in the crystal structure of the yeast *bc*<sub>1</sub> complex with a hydroxy-benzoxythiazol bound at center P [14]. The hydroxyl group of the hydroxy-naphthoquinone binds via a hydrogen bond to the nitrogen of His-181 of the Rieske iron–sulfur protein. On the opposite side of the ring system the carbonyl group at position 4 of the quinone ring of atovaquone forms a water-mediated hydrogen bond with Glu-272 of cytochrome *b*. 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.

There is a high score of cytochrome *b* sequence identity (about 70%) within the atovaquone binding pocket between *S. cerevisiae* and *T. gondii* (Fig. 1A). We have thus chosen the yeast to study how mutations can affect the drug efficacy. Both atovaquone-resistant mutations in the cytochrome *b* sequence previously identified in *T. gondii* were localized within this binding site [13]. These two mutations (M129L and I254L in *T. gondii* numbering, equivalent to M139L and I269L in the yeast numbering system) associated with resistance in *T. gondii* were transferred into the *S. cerevisiae* cytochrome *b* gene 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

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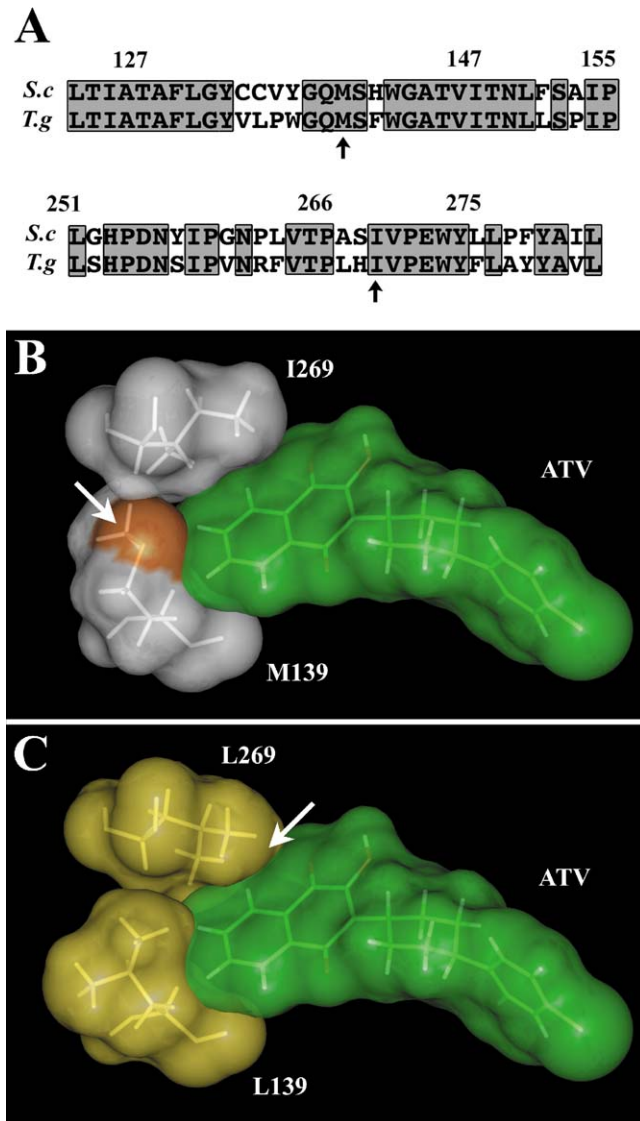


Fig. 1. (A) Sequence alignment of the cytochrome *b*'s of *S. cerevisiae* and *T. gondii* in the conserved regions including residues 123–155 and 251–282 (yeast numbering) around the ubiquinol oxidation pocket at center P. The arrows show the positions of the mutated residues in *T. gondii*. (B and C) Molecular modeling of atovaquone resistant mutations of the *bc*<sub>1</sub> complex from *T. gondii* in *S. cerevisiae*. Only the interactions of the inhibitor with the wild-type (B) and mutated residues (C) are shown. van der Waals radii are represented by orange (sulfur atom of M139), green (atovaquone), white (wild-type residues) or yellow (mutant residues). Arrows in 'B' show the sulfur atom of M139 and steric effect caused by L269 in 'C'. Molecular modeling simulations were carried out as described previously [12].

not severely impair the ubiquinol binding properties of the yeast *bc*<sub>1</sub> complex.

Ubiquinol–cytochrome *c* reductase activities of the purified *bc*<sub>1</sub> 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*<sub>1</sub> complex from wild-type yeast is sensitive to atovaquone with an inhibitor concentration of approximately 25 nM required for 50% inhibition of the activ-

ity of the pure enzyme [7]. Both cytochrome *b* mutations that confer atovaquone resistances to *T. gondii* also alter the IC<sub>50</sub> for inhibition of the yeast *bc*<sub>1</sub> 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<sub>50</sub> value for the pure *bc*<sub>1</sub> complex of 60 nM. The M139L mutation results in a higher level of resistance to the atovaquone, with an IC<sub>50</sub> value of 400 nM, which corresponds to a 16-fold

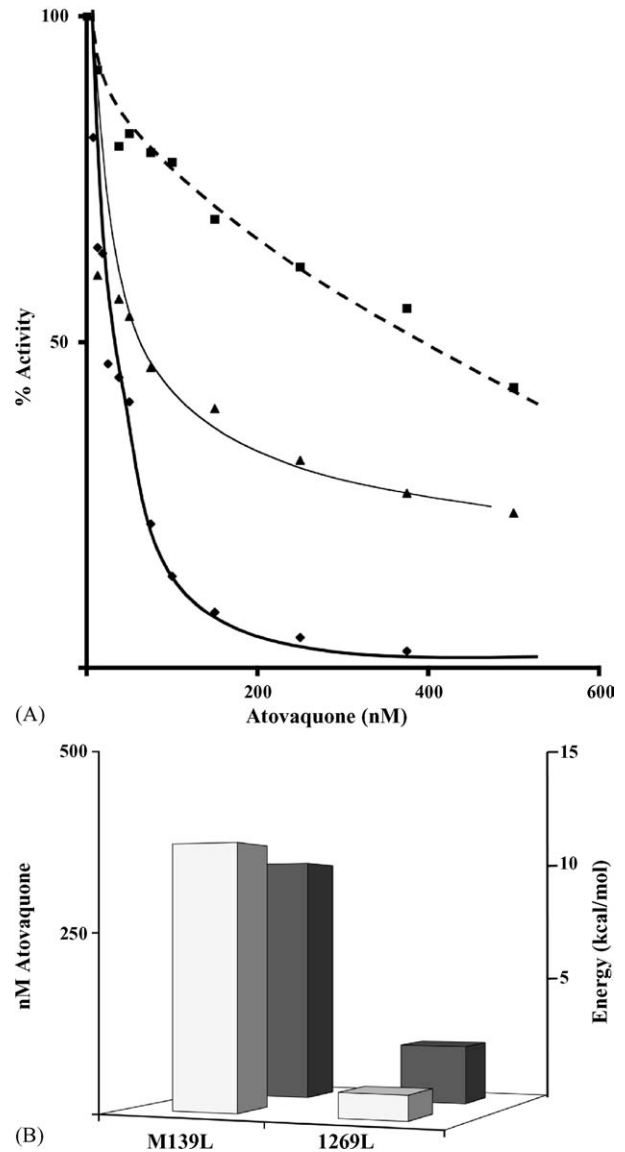


Fig. 2. (A) Relative efficacy of inhibition of wild-type and mutated *bc*<sub>1</sub> complexes by atovaquone. Ubiquinol–cytochrome *c* reductase activities of purified *bc*<sub>1</sub> complexes were measured in the presence of increasing concentrations of atovaquone. Activities are expressed as a percentage of the activity of each *bc*<sub>1</sub> complex in the absence of inhibitor and are indicated as follows: wild-type (diamonds), M139L (squares) and I269L (triangles). (B) Measured changes in atovaquone inhibition and modeled changes in atovaquone binding with atovaquone resistant cytochrome *bc*<sub>1</sub> complexes. The light gray bars indicate the change in IC<sub>50</sub> for inhibition of the *bc*<sub>1</sub> complexes by atovaquone compared to inhibition of the enzyme from wild-type yeast. The dark bars show the calculated change in binding energy for atovaquone with each of the mutated *bc*<sub>1</sub> complexes vs. that with the wild-type enzyme. Inhibition measurements and binding energy calculations were carried out as described previously [12].

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 on 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 IC<sub>50</sub> values. The changes in atovaquone binding energy with the bc<sub>1</sub> complexes from the mutants relative to the binding energy obtained for the wild-type enzyme and the changes in IC<sub>50</sub> 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 IC<sub>50</sub> values for the bc<sub>1</sub> 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 bc<sub>1</sub> 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 energies. With the two current mutations from *T. gondii*, we now have a database of 13 point mutations surrounding the atovaquone binding site. The IC<sub>50</sub> 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.

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