Reconstructing the Qo Site of \textit{Plasmodium falciparum} bc\textsubscript{1} Complex in the Yeast Enzyme

Cindy Vallières\textsuperscript{1*}, Nicholas Fisher\textsuperscript{2}, Brigitte Meunier\textsuperscript{1*}

\textsuperscript{1}Centre de Génétique Moléculaire, Centre National de la Recherche Scientifique, Gif-sur-Yvette, France, \textsuperscript{2}Plant Research Laboratory, Michigan State University, East Lansing, Michigan, United States of America

\section*{Abstract}
The bc\textsubscript{1} complex of the mitochondrial respiratory chain is essential for \textit{Plasmodium falciparum} proliferation, the causative agent of human malaria. Therefore, this enzyme is an attractive target for antimalarials. However, biochemical investigations of the parasite enzyme needed for the study of new drugs are challenging. In order to facilitate the study of new compounds targeting the enzyme, we are modifying the inhibitor binding sites of the yeast \textit{Saccharomyces cerevisiae} to generate a complex that mimics the \textit{P. falciparum} enzyme. In this study we focused on its Qo pocket, the site of atovaquone binding which is a leading antimalarial drug used in treatment and causal prophylaxis. We constructed and studied a series of mutants with modified Qo sites where yeast residues have been replaced by \textit{P. falciparum} equivalents, or, for comparison, by human equivalents. Mitochondria were prepared from the yeast \textit{Plasmodium}-like and human-like Qo mutants. We measured the bc\textsubscript{1} complex sensitivity to atovaquone, azoxystrobin, a Qo site targeting fungicide active against \textit{P. falciparum} and RCQ06, a quinolone-derivative inhibitor of \textit{P. falciparum} bc\textsubscript{1} complex. The data obtained highlighted variations in the Qo site that could explain the differences in inhibitor sensitivity between yeast, plastomidal and human enzymes. We showed that the yeast \textit{Plasmodium}-like Qo mutants could be useful and easy-to-use tools for the study of that class of antimalarials.

\section*{Introduction}
Malaria is among the most serious health problems in the world leading to more than 655,000 deaths in 2010 mainly among children under five years old, according to the World Malaria report 2011. Although several antimalarial drugs have been developed, emerging resistance to many of these compounds and dissemination of drug-resistant \textit{P. falciparum} are compromising the treatment of malaria patients. Thus there is an urgent need for new antimalarial drugs. The complex III or bc\textsubscript{1} complex is an attractive target for antimalarial drugs. This respiratory chain complex is essential for \textit{P. falciparum} proliferation as its catalytic activity is critical for the maintenance of the mitochondrial membrane potential and for the reoxidation of ubiquinol, which is needed for the ubiquinone-dependent dihydroorotate dehydrogenase, and by consequence for the biosynthesis of pyrimidine \cite{1}. In addition, differences in the sequences of the active sites of the bc\textsubscript{1} complex between organisms facilitate the search for inhibitors with selective activity.

The hydroxynaphthoquinone atovaquone is currently the sole bc\textsubscript{1} complex inhibitor used (in combination with proguanil as Malarone\textsuperscript{®}) as antimalarial drug in treatment and causal prophylaxis. The compound is also used to treat \textit{Pneumocystis jirovecii} pneumonia, \textit{Toxoplasma gondii} toxoplasmosis, and other infections. In the USA, Malarone\textsuperscript{®} prescription accounted for more than half of all antimalarial prescriptions. However, the cost of atovaquone is so far prohibitive for more general use. The patent for Malarone\textsuperscript{®} expires this year, which might result in lower cost generics. With the possibility of more extensive use of atovaquone, the risk of resistance to atovaquone would likely increase.

\textit{P. falciparum} atovaquone-resistant parasites have been reported to emerge during atovaquone-proguanil therapy, leading to typical treatment failure \cite{2,3}. The resistance is caused by point mutations in the drug target. Therefore, new drugs that could circumvent the resistance would be required. Different compounds are currently being studied, such as 4(1\textit{H})-pyridones, acridones, acridinediones, and 4(1\textit{H})-quinolones \cite{4} and also \cite{5,6}. Known drugs used to control animal parasites \cite{7} or plant pathogenic fungi \cite{8} have been also revisited. It has been reported, for instance, that the fungicide azoxystrobin that targets the bc\textsubscript{1} complex was a potent inhibitor of \textit{P. falciparum} \cite{8}.

The bc\textsubscript{1} complex contains two sites that bind its substrate quinol and inhibitors, the Q\textsubscript{i} and Q\textsubscript{o} sites. Atovaquone targets specifically the Q\textsubscript{o} site. The Q\textsubscript{i} site and the Q\textsubscript{o} site is provided by the main subunit of the bc\textsubscript{1} complex, cytochrome b. Cytochrome b is mitochondrial encoded in all eukaryotes while the other subunits of the complex are nuclearly encoded. The Q\textsubscript{o} site is a relatively large domain formed from components encompassing amino acid residues 120–130 and 260–280 of the cytochrome b. Hydrogen bonds form a ‘loose stitching’ that allows a degree of expansion of the site on occupant binding \cite{9}. Some sidechains within the Q\textsubscript{o},
site, such as components of the ‘PEYW’ motif (residues 271–274, yeast numbering), demonstrate considerable conformational flexibility on inhibitor binding [10]. The Qc site forms a bifurcated volume. On the basis of structural and mutational analysis, Qc inhibitors can be separated into two classes by their mode of binding [9,10]. For instance, azoxystrobin binds in the so-called ‘haem β proximal’ domain of the Qc site whereas stigmatellin binds in the ‘haem β distal’ domain, occupying the region of the Qc site close to the interface with another catalytic subunit of the complex, the iron-sulphur protein (ISP). Crystal structures for several inhibitor-liganded bc1 complexes are available, such as azoxystrobin- and stigmatellin inhibited enzymes (see for instance, [10–12]). A crystal structure for the atovaquone-inhibited complex is not yet available. However, atovaquone is likely to be a ‘distal’ binding inhibitor due to its effect on the EPR spectrum of the ISP [13]. The quinolone compound RQC06 that showed a promising antimalarial activity might also bind in the ‘distal’ region of the Qc pocket, as suggested by the modelling analysis [14].

In order to facilitate the study of drugs targeting the P. falciparum bc1 complex, we are developing the yeast (Saccharomyces cerevisiae) model. By modifying the inhibitor binding sites of the yeast native bc1 complex, new versions can be generated that mimic the P. falciparum enzyme. In this study, we constructed a series of yeast mutants harbouring variants of the Qc site where yeast residues have been replaced by P. falciparum equivalents, or, for comparison, by the human equivalents. The yeast mutants with Plasmodium-like (and human-like) Qc site were used to investigate the structural determinant of the differential sensitivity to atovaquone and azoxystrobin and to explore the binding mode of the novel molecule RQC06. We also constructed a mutant harbouring the atovaquone resistance mutation Y279S (Y268S in P. falciparum sequence) in the Plasmodium-like enzyme to study the effect of this mutation on new inhibitors such as RQC06. The yeast mutants could be useful tools for the study of new drugs with selective activity and for the analysis of resistance mutations.

Materials and Methods

Materials

Equine cytochrome c, decylubiquinone, azoxystrobin and atovaquone were obtained from Sigma. RQC06 was kindly supplied by Prof. Paul O’Neill (Liverpool, UK).

Yeast Mutants

The mutants were generated by side-directed mutagenesis and mitochondrial transformation as described in [15]. They have identical nuclear and mitochondrial genomes with the exception of the mutations introduced in the cytochrome b gene.

Measurement of Quinol: Cytochrome c Reductase Activity

Yeast mitochondria were prepared as in [16]. Bovine mitochondrial samples were kindly given by Prof. Peter Rich (UCL, UK). Quinol:cytochrome c reductase activity measurements were performed in 10 mM potassium phosphate pH 7 and 20μM equine cytochrome c at room temperature. Mitochondria were diluted to 5–30 nM bc1 complex. Concentrations of monomeric bc1 complex were determined from the cytochrome b a-band in dithionite-reduced optical spectra, using ε = 28.5 mM⁻¹·cm⁻¹ at 562 nm and ε = 557 nm. Activity was initiated by the addition of 20μM decylubiquinol. Cytochrome c reduction was recorded at 550 nm versus 540 nm over a 3-min time-course in a Beckmann DU 640 spectrophotometer. Initial rates were measured. From these rates, turnover numbers (TN) were calculated as cytochrome c reduced per bc1 complex per second.

Inhibitor Titration

Cytochrome c reduction activity was measured as described above in presence of increasing concentrations of inhibitors (six to ten different concentrations). Each measurement was repeated at least twice and averaged. The errors did not exceed 10%. The mid-point inhibition concentrations (IC50) were determined from the titrations. As the titrations were performed using mitochondrial samples containing different concentrations of bc1 complex (5–30 nM), the results were presented as ratio of IC50 on the concentration of bc1 complex.

Ligand Docking and Molecular Modelling

Atovaquone was docked into the Qc site of yeast cytochrome b (3CX5.PDB) as described in [17]. An atomic model of RQC06 was created using PRODRG2 and the Dock Prep module of Chimera [18,19]. The energy-minimised RQC06 model was docked into a 9Å radius sphere centred on the ε2-oxygen atom of cytochrome b residue E272 in the Qc site of 3CX5.PDB using EADock DSS via SWISSDOCK [20]. Using an iterative search, ligand binding modes with favourable CHARMM energies were clustered taking account of the solvent effect with the FACTS implicit solvation model, and the resulting output files examined with Chimera and VMD.

Results and Discussion

1) Sensitivity to Qc Inhibitors: Comparison between Plasmodial, Mammalian and Yeast bc1 Complexes

The sensitivity of the bc1 complex (cytochrome c reductase) activity towards atovaquone, azoxystrobin and RQC06 is presented in Table 1. As previously reported, yeast bc1 complex, as P. falciparum enzyme, is highly sensitive to atovaquone (IC50 of 4 (molar ratio)) while the bovine enzyme was less reactive (IC50 of 75). Azoxystrobin has been shown to be a potent inhibitor of P. falciparum proliferation with an IC50 in the nanomolar range (15 nM [8]). The drug inhibits yeast bc1 complex with an IC50 of 20 while it is less active against the bovine enzyme (IC50 of 180). RQC06 was also reported as potent inhibitor of the plasmodial bc1 complex [14] with an IC50 of around 1.3 nM, which is in the same range than the atovaquone IC50 (around 3 nM) [21]. We found that the bovine enzyme was less sensitive to that compound (IC50 of 40). The differential sensitivities to RQC06 and atovaquone between the Plasmodium and mammalian enzymes are thus similar.

By contrast, the yeast enzyme was highly resistance to the RQC06 (IC50>500).

In order to study the structural determinants that could explain the differential sensitivity to these drugs, we monitored the impact of mutations introduced in the Qc site of the yeast bc1 complex, single mutations and combined Plasmodium- or human-like changes.

2) Determinants of Atovaquone Sensitivity Studied in Plasmodium-like Yeast Qc Site Mutants

In previous works, the low atovaquone sensitivity of the bovine bc1 complex (as compared to the sensitivity of the yeast enzyme) was investigated. A computed energy-minimised structure for atovaquone liganded to the yeast bc1 complex suggested that residue 275 plays a key role in the differential sensitivity. The presence of a phenyalanine at position 275 in the bovine enzyme would hinder the drug binding. F275 being replaced by L in the yeast enzyme, the steric constraint is lessened and the sensitivity to atovaquone increases. When the mutation L275F was genetically
introduced in the yeast bc\(_1\) complex, its sensitivity was significantly decreased (over 20-fold) [13,15,22].

In the cytochrome \(b\) of Plasmodium falciparum and of other Plasmodium and of Toxoplasma, the F275 variant is naturally present. However, the parasite \(bc\)\(_1\) complex is highly sensitive to atovaquone. Thus other structural variations should explain the differential sensitivity between the parasite and the mammalian enzymes.

Comparison of the cytochrome \(b\) sequences (Figure 1A) shows that the \(Q_o\) domain is well conserved between organisms. There are however variations that may affect atovaquone susceptibility.

In order to investigate their impact, we constructed a series of yeast strains harbouring multiple changes in the \(Q_o\) domain. We modified the \(bc\)\(_1\) complex to inhibit the quinol cytochrome \(bc\)\(_1\) complex to inhibit the quinol cytochrome \(bc\)\(_1\) complex of Toxoplasma gondii and \(P\). jiroveci are located at positions 139, 147, 148, 150, 152, 266, 269, 275, 278, 279, 282, 283, 291 and 295 [23–29].

Most residues are conserved in yeast and \(P\). falciparum, with the exception of residues 275, 283 and 295. Thus the Plasmodium-like mutations studied here are located at residues or closed to residues involved in acquired-atovaquone resistance.

The \(Q_o\) pocket of PF11 best mimics \(P\). falciparum \(Q_o\) site as it harbours most of the variations that might affect the susceptibility to atovaquone or other \(Q_o\) site inhibitors. The combined change was well tolerated: the \(bc\)\(_1\) complex retained more than 40% activity (Table 1).

Interestingly, PF11 was highly sensitive to atovaquone despite the presence of F275 (IC\(_{50}\) of 3) whereas F275 alone in the yeast \(Q_o\) site (mutant L275F) caused a strong atovaquone resistance (IC\(_{50}\) of 150). A similar behaviour was observed with PF2 and PF8 but the atovaquone sensitivity was less marked (IC\(_{50}\) of 10 and 16, respectively). In Rhodobacter capsulatus cytochrome \(b\), a phenylalanine is naturally present at this position. The IC\(_{50}\) of atovaquone was estimated to be around 30 nM. Interestingly, the sensitivity of the bacterial complex to atovaquone was increased (IC\(_{50}\) decreasing from 30 nM to 10 nM) by the introduction of two mutations replacing bacterial residues by the plasmodial equivalents, namely I281M and R283K (yeast numbering) [30], which is in agreement with our data as R283K is present in PF8. It seems therefore that when L275F is combined to other Plasmodium-like mutations, the impact of L275F is weakened. From these data, it appears that the atovaquone sensitivity of \(P\). falciparum \(bc\)\(_1\) complex could be explained by a combination of variations (variations from the yeast \(Q_o\) site) that cancel the resistance effect of F275. We could hypothesise that the \(P\). falciparum \(Q_o\) site and the yeast Plasmodium-like \(Q_o\) site have a larger volume that could accommodate atovaquone despite a phenylalanine in position 275. The modified yeast \(bc\)\(_1\) complex harbouring a Plasmodium-like \(Q_o\) and highly susceptible to atovaquone (PF11) could be useful model to study the effect of resistance mutations.

### Table 1. \(bc\)\(_1\) complex activity and sensitivity to \(Q_o\) site inhibitors.

| \(bc\)\(_1\) complex source | TN (%) | IC\(_{50}\) atovaquone | IC\(_{50}\) azoxystrobin | IC\(_{50}\) RCOQ6 |
|---------------------------|-------|----------------------|------------------------|------------------|
| P. falciparum             |         | 3 nM                 | nd                     | 1.3 nM           |
| bovine                    |         |                      |                        |                  |
| Yeast WT                  | 100    | 75                   | 180                    | 40               |
| Plasmodium-like mutants   |         |                      |                        |                  |
| PF1                       | 52     | 2                    | 5                      | >500             |
| PF2                       | 105    | 10                   | 16                     | 10               |
| PF8                       | 107    | 16                   | 220                    | 40               |
| PF11                      | 44     | 3                    | 40                     | 25               |
| PF12                      | 15     | >850                 | 30                     | >500             |
| Y279S                     | 27     | >850                 | 60                     | >500             |
| Human-like mutants        |         |                      |                        |                  |
| L275F                     | 110    | 150                  | 30                     | 10               |
| F278A                     | 61     | 35                   | 120                    | –                |
| M295L                     | 120    | 10                   | 45                     | –                |
| HSa                       | 81     | 200                  | 130                    | –                |
| HSb                       | 47     | 170                  | 160                    | –                |
| HSc                       | 89     | 60                   | 140                    | –                |

The mutated residues in the yeast strains are presented in Table 2 for the Plasmodium-like mutants (PF) and in Table 3 for the human-like mutants (HS). Their location in the sequence and the structure is shown in Fig. 1. TN, turnover number: cytochrome \(c\) reduced per \(bc\)\(_1\) complex per second (Materials and Methods). The values are presented as % of the WT activity (140 s\(^{-1}\)).

IC\(_{50}\), mid-point inhibition concentration. The IC\(_{50}\) values for bovine and yeast enzymes were obtained as described in Materials and Methods. The values are presented as ratio of IC\(_{50}\) on the concentration of monomeric \(bc\)\(_1\) complex (estimated using cytochrome optical signal as in Materials and Methods). For example, 4 molecules of atovaquone were added per yeast WT monomeric \(bc\)\(_1\) complex to inhibit the quinol cytochrome \(c\) redoxactase activity by 50%.

For \(P\). falciparum enzyme, the atovaquone IC\(_{50}\) is taken from [21]. In the same study, the IC\(_{50}\) for bovine and human enzymes were approximately 70 nM. The IC\(_{50}\) value from [14]. Note that the \(bc\)\(_1\) complex concentrations in the \(P\). falciparum preparations used for inhibitor titrations were not available in the published studies. It was thus not possible to present the data as the molar ratio IC\(_{50}\)/\(bc\)\(_1\) complex. An IC\(_{50}\) of azoxystrobin is not available. The inhibitor was reported to be highly active on the parasite growth [8].

The mammalian \(bc\)\(_1\) complex is naturally resistant to atovaquone (as compared to the plasmodial and the yeast enzymes) (Table 1).

To investigate further the observed resistance, we modified the \(Q_o\) site of yeast \(bc\)\(_1\) complex and introduced human variations, single or combined (Table 3). The substitutions were well accommodated in yeast \(Q_o\) site. The mutant enzyme combining eight human changes (HS) showed nearly WT activity (89%).

The three human-like mutations, L275F, F278A and M295L, conferred some level of resistance towards atovaquone. The IC\(_{50}\) increased 35-, 9- and 2.5-fold in L275F, F278A and M295L, respectively. The combination of these mutations (HSa and HSb)
caused a further increase of resistance. When combined with amino-acid changes at positions 133 to 141 (HSc), the resistance to atovaquone was decreased, as observed with *Plasmodium*-like mutants. However, the human-like *bc*₁ complex remained 15-fold more resistant than the WT enzyme, with an IC₅₀ value of 60, similar to the IC₅₀ value of 75 obtained with the bovine enzyme. It can thus be suggested that the combination of F275, A278 and L295 in human *bc*₁ complex is responsible for the natural resistance towards atovaquone.

4) Sensitivity and Resistance to Azoxystrobin of *Plasmodium*- and Human-like Mutants

In the human-like mutants, F278A caused a 6-fold increase in azoxystrobin resistance, M295L and L275F had a weaker effect. The triple mutation (HSb) conferred 8-fold resistance. Changes in position 133 to 141, when combined with that triple mutation (HSc), did not affect the resistance (IC₅₀ of 140). Thus the 9-fold resistance of the mammalian enzyme to azoxystrobin (IC₅₀ of 180), compared to yeast enzyme (IC₅₀ of 20) could be explained by the combination of F275, A278 and L295 in the binding site.

Figure 1. Sequence and structure of the Q₀ site. (A) Comparison of cytochrome b sequence. Regions of the polypeptide forming the Q₀ domain and its vicinity are shown. The Q₀ site itself is formed by residues located in the regions 120–150 and 260–280. Mutated residues studied here are highlighted in colour. Green arrows and bars indicate structural components, as shown in panels B and C: C and F₁ are transmembrane helices; cd₁ and cd₂, extramembrane short helices; ef, a loop containing highly conserved residues. Hs, human; Sc, yeast; Pf, *Plasmodium falciparum*. (B) Location of the mutated residues in the yeast *bc*₁, Q₀ site. The figure was drawn using the coordinates of 3CX5.PDB [39]. VLPW, location of the mutation CCYV₁₃₃₋₁₃₆VLPW. (C) Molecular model of atovaquone (lilac CPK) and RCQ06 (cyan CPK) docked in the Q₀ site of yeast cytochrome b (3CX5.PDB [39]). Selected sidechains from cytochrome b and the ISP are represented in orange. The alpha carbon backbones of cytochrome b and the ISP are represented in cartoon form in grey/green and dark pink respectively. Helices of interest within cytochrome b forming structural elements of Q₀ are labelled in green. Also shown are the haem b₁ and [2Fe2S] prosthetic groups of cytochrome b and the ISP. Residue notation corresponds to the yeast enzyme. Atv, atovaquone.

doi:10.1371/journal.pone.0071726.g001
In the *Plasmodium*-like yeast mutants, modifications of residues 283 to 299 caused a 10-fold increase in resistance (IC₅₀ of 220 for PF8), but the changes in position 133 to 141 lowered the resistance by 5 fold (IC₅₀ of 40 for PF11). That combination of residues might explain the susceptibility of *P. falciparum* to azoxystrobin.

As a control, we compared the sensitivity to the Qᵯ site inhibitor antimycin of the bc₁ complex of the human- and the *Plasmodium*-like mutants, HSc and PF11, with that of the WT control. No or very little difference was observed (data not shown).

5) Effect of the Acquired Atovaquone Resistance Mutation Y279S (Y268S in *P. falciparum*)

In *P. falciparum* isolated from patients after treatment failure, three mutations have been reported, Y279S/C/N (Y268 in *P. falciparum*). Mutations Y279S and C are the most frequent mutations in the parasite. Their consequences on the catalytic activity and the stability of the complex have been characterized in the *P. falciparum* (Y268S) [17], the yeast [31,32] and the bacterial enzyme [30,33].

We introduced the mutation Y279S in PF11 to obtain a model mimicking *P. falciparum* resistant enzyme. As presented in Table 1, the resulting mutant enzyme (PF12) showed a more severe decrease in activity, retaining only 15% of the WT activity. This was expected as the mutation Y279S alone in the yeast Qᵯ complex caused a 70% decrease in the bc₁ complex activity. In the parasite bc₁ complex, the acquired resistance mutation resulted in a low activity and unstable ISP [17]. It has been suggested that residue Y279 is involved in the correct orientation of the quinol bound in the Qᵯ site via tyrosyl-benzoquinone hydrophobic packing, facilitating quinol deprotonation and electron transfer to the ISP [34]. Substitution of the tyrosine by (the less bulky) serine is likely to abolish, or otherwise weaken, this predicted stabilising interaction interfering with optimal quinol binding, potentially slowing the reduction of the ISP [2Fe2S] cluster redox group.

When introduced in the *Plasmodium*-like Qᵯ site (PF12) or in the yeast Qᵯ site, Y279S caused a high level of resistance to atovaquone (IC₅₀>850), similar to that observed with the Y268S plasmodial enzyme. By contrast, the sensitivity to azoxystrobin was little affected by the atovaquone resistance mutation (IC₅₀ of 40 and 30 for PF11 and PF12, respectively). This result was not unexpected since azoxystrobin has been shown to bind in the Qᵯ site at a position proximal to the haem b₁ [10], while atovaquone binds at a position distal to the haem b₁ [13]. However it emphasizes the interest of developing molecules, such as azoxystrobin, that have both differential reactivity and can circumvent the atovaquone-resistance mutation.

6) RCQ06 Binding, Comparison with Atovaquone

As shown in Table 1, the yeast bc₁ complex is highly resistant to RCQ06. Strikingly, the introduction of mutation L275F rendered the enzyme over 50-fold more sensitive to RCQ06 than WT. The sensitivity to RCQ06 was observed in all the mutants with L275F in the Qᵯ site: PF2, PF8, and PF11. Thus RCQ06 binding is likely to be stabilised by an aromatic residue at position 275, whereas the binding of atovaquone is hindered by the bulkier residue. The chemical structures of RCQ06, atovaquone and azoxystrobin are presented in Figure 2.
The introduction of the atovaquone resistance mutation Y279S in Plasmodium-like Qo site (PF12) caused a high level of resistance to RCQ06, as it did for atovaquone. This suggests that the two inhibitors have similar binding mode. On the basis of this mutational analysis, a model of RCQ06 has been build. Figure 1C presents a molecular model of atovaquone and RCQ06 docked into the Qo site of the yeast bc1 complex. As mentioned in the introduction, a crystal structure of the atovaquone-bound enzyme is not available but atovaquone is predicted to interact with the Qo site in a manner similar to that of stigmatellin via hydrogen bonding from its naphthoquinone moiety via a bridging water molecule to E272 (yeast numbering) of cytochrome b, with a second hydrogen bond formed to the imidazole group of the ISP [2Fe2S] cluster ligand H181 [13].

Our model suggests that RCQ06 binds in a manner reminiscent to that of stigmatellin with a 3.5Å hydrogen bond between its quinolone amine moiety and the glutamyl sidechain of cytochrome b residue E272. This association would require that the glutamyl is acting as the hydrogen bond acceptor, and is thus present in the deprotonated (carboxylate) form, which is thermodynamically accessible when haem b is oxidised [35]. In the docking model, the quinolone carbonyl of RCQ06 is within hydrogen bonding distance of [2Fe2S] cluster ligand H181 (2.9Å), although the distance of [2Fe2S] cluster ligand H181 (2.8Å), although the prediction of sensitivity/resistance phenotype associated with formation of such a hydrogen bond would require the histidyl sidechain of this residue to be present as a protonated imidazole, favouring the reduced form of the [2Fe2S] cluster. Perhaps surprisingly, we predict no hydrogen bonding interactions between the trifluoromethoxy group of RCQ06 and the surrounding protein. A stabilising aliphatic/aromatic interaction (2.7Å) is predicted to occur between the sidechain of L275 and the quinolone moiety of RCQ06. As shown in Table 1, the experimental data indicate that the determinant of the sensitivity towards RCQ06 is a phenylalanine in position 275, which is naturally present in plasmodial and mammalian cytochrome b. Inspection of the model presented in Figure 1C suggests that a favourable aromatic/aromatic interaction may be formed between L275 and the RCQ06 quinolone group, with a larger van der Waals contact area than that observed for the proposed L275/RCQ06 interaction, effectively packing this sidechain against the inhibitor. A similar stabilising aromatic/aromatic interaction, with the aromatic groups oriented perpendicularly, is predicted to form between the sidechain of Y279 and the quinolone moiety of RCQ06. Replacement of Y279 by a serine would destabilise RCQ06, which would explain the observed resistance.

Conclusions

Acquired resistance to atovaquone developed readily, which is caused by the mutation of the cytochrome b residue Y279 (Y268 in P. falciparum). New drugs are needed that circumvent the acquired atovaquone resistance: for example, drugs targeting the Qo site [36,37] and compounds binding at the Qo site in the haem bc1-proximal region such as azoxystrobin or in the distal site but with different binding interactions than atovaquone [38].

Cytochrome b sequences from yeast, human and P. falciparum are highly conserved, making yeast an attractive model to investigate sensitivity and resistance to drugs targeting the bc1 complex. Crystal structures of yeast bc1 complex are available. Most importantly, genetic tools have been well developed to introduce designed mutations in yeast mitochondrially-encoded cytochrome b gene. Yeast is the only organism, with the green algae Chlamydomonas reinhardtii, amenable to mitochondrial transformation. By modifying the yeast enzyme, useful tools could be created for the discovery and the analysis of new drugs with potential antimalarial activity and for the study of the development of resistance mutations.

In previous reports, we studied acquired resistance mutations found in parasites and introduced in yeast Qo site [15,22,32]. In this study, we generated yeast mutants with modified Plasmodium-like Qo sites that mimic the site of the plasmodial enzyme. We tested the sensitivity to atovaquone and azoxystrobin to validate the model. We showed that the differential sensitivity to atovaquone and azoxystrobin, and the high resistance caused by Y279S (Y268S) could be reproduced in the yeast models.

We then used the mutants to study the binding mode of a new molecule RCQ06. The experimental data, together with the model of RCQ06 binding in the Qo site, indicated that the drug shares the same binding pocket than atovaquone. Even though the binding mode of RCQ06 might not be identical to that of atovaquone, the efficiency of binding is affected by the Y279S mutation in the yeast enzyme.

More remodelling of the yeast Qp and Qo sites will be performed to obtain easy-to-use models that mimic more accurately the plasmodial enzyme. These mutants with a P. falciparum-like enzyme could be useful tools for the discovery and the analysis of new drugs with potential anti-malarial activity and for the prediction of sensitivity/resistance phenotype associated with
naturally occurring cytochrome \( b \) polymorphisms segregating in parasite populations.

References

1. Painter HJ, Morrisey JM, Mather MW, Vaidya AB (2007) Specific role of mitochondrial electron transport in blood-stage *Plasmodium falciparum*. Nature 446: 88–91.

2. Misset L, Bouchaud O, Matheron S, Massias L, Le Bras J (2006) Clinical atovaquone-proguanil resistance of *Plasmodium falciparum* associated with cytochrome \( b \) codon 261 mutations. Microbes Infect 8: 2599–2604.

3. Misset L, Le Bras J, Clain J (2007) Parallel evolution of adaptive mutations in *Plasmodium falciparum* mitochondrial DNA during atovaquone-proguanil treatment. Mol Biol Evol 24: 1382–1385.

4. Bartov V, Fisher N, Bagiñi GA, Ward SA, O’Neill P (2010) Inhibiting *Plasmodium* cytochrome bc\(_1\): a complex issue. Curr Opin Chem Biol 14: 1–7.

5. Bagiñi GA, Fisher N, Shone AE, Mubaraki MA, Srivasta A, et al. (2012) Generation of quinolone antimalarials targeting the *Plasmodium falciparum* mitochondrial respiratory chain for the treatment and prophylaxis of malaria. Proc Natl Acad Sci U S A 109: 8296–8303.

6. Nilsen A, Lacrue A, White KL, Forquer IP, Cross RM, et al. (2013) Generating human infection-relevant *Toxoplasma gondii* strains as models of biological and pathogenic processes. PLoS Negl Trop Dis 6: e1939.

7. Nam TG, McNamara GW, Bopp S, Dharia NV, Meister S, et al. (2011) A human disease-related mutation in cytochrome \( b \) confers resistance to atovaquone-proguanil. Mol Biochem Parasitol 177: 1349–1363.

8. Walker DJ, Wakefield AE, Dohn MN, Miller RF, Baugman RP, et al. (1998) Sequence polymorphisms in the *Plasmodium falciparum* cytochrome \( b \) gene and their association with atovaquone prophylaxis failure. J Infect Dis 178: 1767–1775.

9. Srivasta IK, Morrisey JM, Darrouzet E, Daldal F, Vaidya AB (1999) Resistance mutations reveal the atovaquone-binding domain of cytochrome \( b \) in malaria parasites. Mol Microbiol 33: 704–711.

10. Christy JA, Durairajan Y, Almeida LH, Sabau LA, Almeida AP, et al. (2001) Resistances to antimalarials are located at a putative drug-binding site. Antimicrob Agents Chemother 44: 2100–2108.

11. Lemaire C, Dujardin G (2008) Preparation of respiratory chain complexes from *Pneumocystis carinii*. J Biol Chem 283: 17542–17549.

12. Grosdidier A, Zoete V, Michelin O (2011) SwissDock, a protein-small molecule docking web service based on EADock DSS. Nucleic Acids Res 39: 270–277.

13. Kessl JI, Hill P, Lange BB, Merbitz-Zahradnik T, Zwicker K, et al. (2003) Recapitulation of the cytochrome \( bc_1 \) complex: a new model system. J Biol Chem 278: 31312–31318.

14. Barmada MM, Dwivedi A, Rokaya B, Kerber R, Paul N, et al. (2007) A resolution of the cytochrome \( bc_1 \) complex from *Saccharomyces cerevisiae*. J Biol Chem 283: 17542–17549.

Author Contributions

Conceived and designed the experiments: NF BM. Performed the experiments: CV NF. Analyzed the data: CV NF BM. Contributed reagents/materials/analysis tools: CV NF BM. Wrote the paper: NF BM.