Agents antimicrobiens ciblant le complexe III de la chaine respiratoire mitochondriale : Etudes des déterminants structuraux de la sensibilité différentielle et du développement de la résistance, en utilisant la levure comme organisme modèle

Zehua Song

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M. Zehua SONG

Agents antimicrobiens ciblant le complexe III de la chaîne respiratoire mitochondriale : Études des déterminants structuraux de la sensibilité différentielle et du développement de la résistance

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Composition du Jury :

Mme, REBOUD-RAVAUX, Michèle	PR. Université Pierre et Marie Curie	Présidente
Mme, ZITO, Francesca	CR, IBPC-CNRS	Rapporteuse
Mme, REMACLE, Claire	PR, Université de Liège	Rapporteuse
M, PICOT, DANIEL	DR, IBPC-CNRS	Examinateur
M, IORGA, Bogdan	CR, ICSN-CNRS	Examinateur
Mme, MEUNIER, Brigitte	CR, I2BC-CNRS	Directrice de thèse

Titre :

Agents antimicrobiens ciblant le complexe III de la chaîne respiratoire mitochondriale: Étude des déterminants structuraux de la sensibilité différentielle et du développement de la résistance, en utilisant la levure comme organisme modèle

Mots clés :

mitochondrie, complexe III respiratoire, mutation de résistance, modèle levure, drogues antipaludique, polymorphisme humain

Résumé :

Le complexe bc_1 de la chaîne respiratoire mitochondriale est une bonne cible thérapeutique pour traiter le paludisme car cette enzyme est essentielle au parasite. Ses deux sites actifs, Q_o et Q_i, formés par le cytochrome *b*, ne sont pas totalement conservés entre les espèces, facilitant la découverte d'inhibiteurs à affinité différentielle, ce qui est important dans le développement de médicaments. L'atovaquone est le seul antipaludique ciblant le complexe bc_1 utilisé en médecine. L'émergence de résistance rend urgente l'étude de nouveaux inhibiteurs. Les ELQs (Endochin-like Quinolones) sont une classe d'antipaludiques particulièrement prometteuse.

Pour étudier la liaison des inhibiteurs dans les sites actifs et l'effet de mutations de résistance, nous utilisons la levure et des méthodes biochimiques et bio-informatiques. Dans ce travail, nous avons étudié la relation entre mutations de résistance à l'atovaquone dans le site Q_o et perte de fonction. Nous avons aussi modifié le site Q_o de la levure pour qu'il mime mieux le site de l'enzyme du parasite. Les résidus «*Plasmodium*» altèrent le fonctionnement du site, résultant en une surproduction d'ions superoxides et une perte de croissance respiratoire, qui est restaurée par la modification d'une autre sous-unité du complexe, ISP, partenaire du site Q_o, suggérant que les deux sous-unités doivent s'ajuster pour un fonctionnement correct. Nous avons analysé des polymorphismes de la région Q_o observés chez l'Homme et trouvé qu'ils peuvent modifier la sensibilité du complexe à l'atovaquone, ce qui pourrait avoir un impact sur les effets secondaires du traitement. Nous avons ensuite étudié le mode d'action d'ELQ-400 et montré que ce nouvel antipaludique cible les deux sites Q_o et Q_i, ce qui rend l'apparition de résistance peu probable. Enfin, nous avons commencé la reconstruction du site Q_i de la levure pour mimer le site du parasite.

Les mutants de levure avec un complexe bc_1 «*Plasmodium*» semblent être de bons outils pour l'étude des inhibiteurs. Leur étude a aussi permis de comprendre mieux la structure et le fonctionnement du complexe bc_1.

Title:

Anti-microbial agents targeting complex III of the mitochondrial respiratory chain: Studying the structural determinants of differential sensitivity and the development of resistance, using yeast as a model organism.

Keywords :

Mitochondrion, respiratory complex III, resistance mutation, yeast model, antimalarial drugs, Human polymorphism

Abstract :

The *bc*$_1$ complex of the mitochondrial respiratory chain is a good therapeutic target for the treatment of malaria as the enzyme is essential for pathogen proliferation. The two catalytic sites, Q_o and Q_i, formed by cytochrome *b*, are not fully conserved between species, facilitating the development of inhibitors with differential saffinity, which is important for the development of new drugs. At present, Atovaquone is the only antimalarial drug targeting the *bc*$_1$ complex used in medicine. The emergence of resistance makes it important to find new inhibitors, and the ELQs (Endochin-like Quinolones) are promising antimalarial candidates.

In order to study the inhibitor binding to the active sites and the effect of resistance mutations, we have used yeast and a combination of biochemical and bioinformatic methods. We have studied the relationship between atovaquone resistance mutations in the Q_o site and loss of function. We have also modified the yeast Q_o site to make it more like the parasite site. The "*Plasmodium*" residues in the yeast Q_o site altered its activity, which resulted in the overproduction of superoxide and the loss of respiratory growth. This could be restored by the modification of another *bc*$_1$ complex subunit interacting with the Q_o site, ISP, suggesting that both these subunits need to be readjusted for correct activity. We then analyzed polymorphisms of the Q_o region reported in Humans and found that they could alter the enzyme sensitivity to atovaquone, which could impact the side-effects linked to atovaquone treatment. We have also studied the mode of action of ELQ-400 and showed that this new antimalarial drug targets both the Q_o and Q_i sites, which would make the emergence of resistance less likely. Finally, we have started the reconstruction of yeast Q_i site to make it resemble the parasite site.

The yeast mutants with a "*Plasmodium*-like" *bc*$_1$ complex could be useful tools for the study of antimalarial drugs. These analyses have also resulted in a better understanding of the structure and function of the *bc*$_1$ complex.

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Chargée de recherche à l'Institut de Biologie Physico-Chimique

Mme Claire REMACLE

Professeur à l'Université de Liège

Mr Daniel PICOT

Directeur de recherche à l'Institut de Biologie Physico-Chimique

Mr Bogdan I. Iorga

Chargé de recherche à l'Institut de Chimie des Substances Naturelles

Mme Michèle REBOUD-RAVAUX

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Abréviations

ATP/ADP : Adénosine TriPhosphate/DiPhosphate

ARN : Acide RiboNucléique

ADN : Acide DésoxyriboNucléique

CCCP : Carbonyle Cyanide 3-ChloroPhenylhydrazone

CDL : Cardiolipine

CLA : chlorophylle

CoA : Coenzyme A

CoQ : Coenzyme Q ou ubiquinol/ubiquinone

Cytb : cytochrome *b*

EI : Espace Intermembranaire

ELQ : Endochin qui ressemble à la quinolone (en anglais : Endochin-Like Quinolone)

$FADH_2$: Flavine Adénine Dinucléotide réduite

IC_{50} : Concentration nécessaire d'un inhibiteur pour inhiber 50% d'activité enzymatique

ISP : Protéine fer-soufre (en anglais : Iron Sulphur Protein)

K_M : Constante de Michaelis

NADH: Nicotinamide Adénine Dinucléotide Hydride

NQNO : 2-nonyl-4-hydroxyquinoline-N-oxide

OD: densité optique (en anglais: Optical Density)

OxPhos : Phosphorylation oxydative

PCR : Réaction en chaîne par polymérase (en anglais : Polymérase Chain reaction)

PDB: Banque de données des protéines (en anglais: Protein Data Bank)

ROS : Espèce réactive d'oxygène (en anglais : Reactive Oxygen Species)

SO : SuperOxide

SOD : SuperOxide Dismutase

TN : constante catalytique (en anglais : Turnover Number)

wt : la souche sauvage (en anglais : wild-type)

[2Fe-2S] : cluster fer-soufre

Introduction générale

Introduction Générale

A. La levure *Saccharomyces cerevisiae* comme modèle d'organisme

Pendant cette thèse, j'ai utilisé la levure de boulanger *Saccharomyces cerevisiae* comme modèle d'organisme pour étudier le complexe bc_1 de la chaîne respiratoire mitochondriale, en particulier le complexes bc_1 du pathogène *Plasmodium falciparum*, cible d'agents antipaludiques.
La levure présente plusieurs avantages pour ce type d'étude :

- Plusieurs structures cristallographiques du complexe bc_1 de levure avec des inhibiteurs différents sont disponibles (Tableau 2), tandis que la structure n'existe ni pour l'enzyme de humaine ni pour celle de *Plasmodium* à ce jour.
- Pour étudier le rôle de certains résidus et de certaines mutations de résistance aux inhibiteurs, nous devons modifier les gènes codant pour les sous-unités du complexe bc_1. Mais une des sous-unités catalytiques, le cytochrome *b*, est codé par le génome mitochondrial. La seule technique qui permet de faire de la mutagénèse dirigée dans un gène mitochondrial (la transformation biolistique) est actuellement possible seulement chez la levure *S. cerevisiae* et l'algue *Chlamydomonas reinhardtii*.
- La levure est capable de croître par fermentation sans utiliser sa chaîne respiratoire pour produire de l'énergie. Cette propriété est particulièrement utile pour l'étude de mutants avec des mutations rendant leur complexe bc_1 peu ou non fonctionnel.
- Le coût pour obtenir la même quantité de biomasse est considérablement plus bas pour la levure que pour le parasite. Les procédures d'extraction de mitochondrie est beaucoup plus simple.
- Divers outils génétiques sont disponibles et d'utilisation relativement facile.
- La séquence (et donc la structure) du cytochrome *b*, la sous-unité qui contient les deux sites de liaison des substrats du complexe bc_1, est bien conservée entre les espèces. La Figure 1 présente la comparaison de l'identité des séquences du cytochrome *b* de cinq espèces : *S. cerevisiae*, *P. falciparum*, *Homo sapiens*, *Bos Taurus* et *Gallus gallus*. Le pourcentage d'identité de la séquence du cytochrome *b* est présenté dans le Tableau 1. Les structures atomiques des enzymes de *B. Taurus* et *G. gallus* sont aussi disponibles. La séquence du cytochrome *b* de levure a 38% et 50% d'acides aminés identiques à celle de *P. falciparum* et de *l'Homo Sapiens*, respectivement. Les résidus qui participent à la formation des deux sites catalytiques sont encore mieux conservés, en particulier les résidus qui participe à la formation du site Q_o : le pourcentage de similarité peut aller jusqu'à 86%.

Tableau 1. La matrice de comparaison d'identité en pourcentage de la séquence du cytochrome *b* entre les cinq espèces.

	S. c.	***P. f.***	***H. s.***	***B. t***	***G. g.***
S. c.	100.00	35.95	49.34	51.06	51.59
P. f.	35.95	100.00	42.08	42.19	41.37
H. s.	49.34	42.08	100.00	78.89	72.56
B. t	51.06	42.19	78.89	100.00	74.67
G. g.	51.59	41.37	72.56	74.67	100.00

S. c. : *Saccharomyces cerevisiae, P. f.* : *Plasmodium falciparum, H. s.* : *Homo sapiens, B. t.* : *Bos Taurus, G. g.* : *Gallus gallus.*

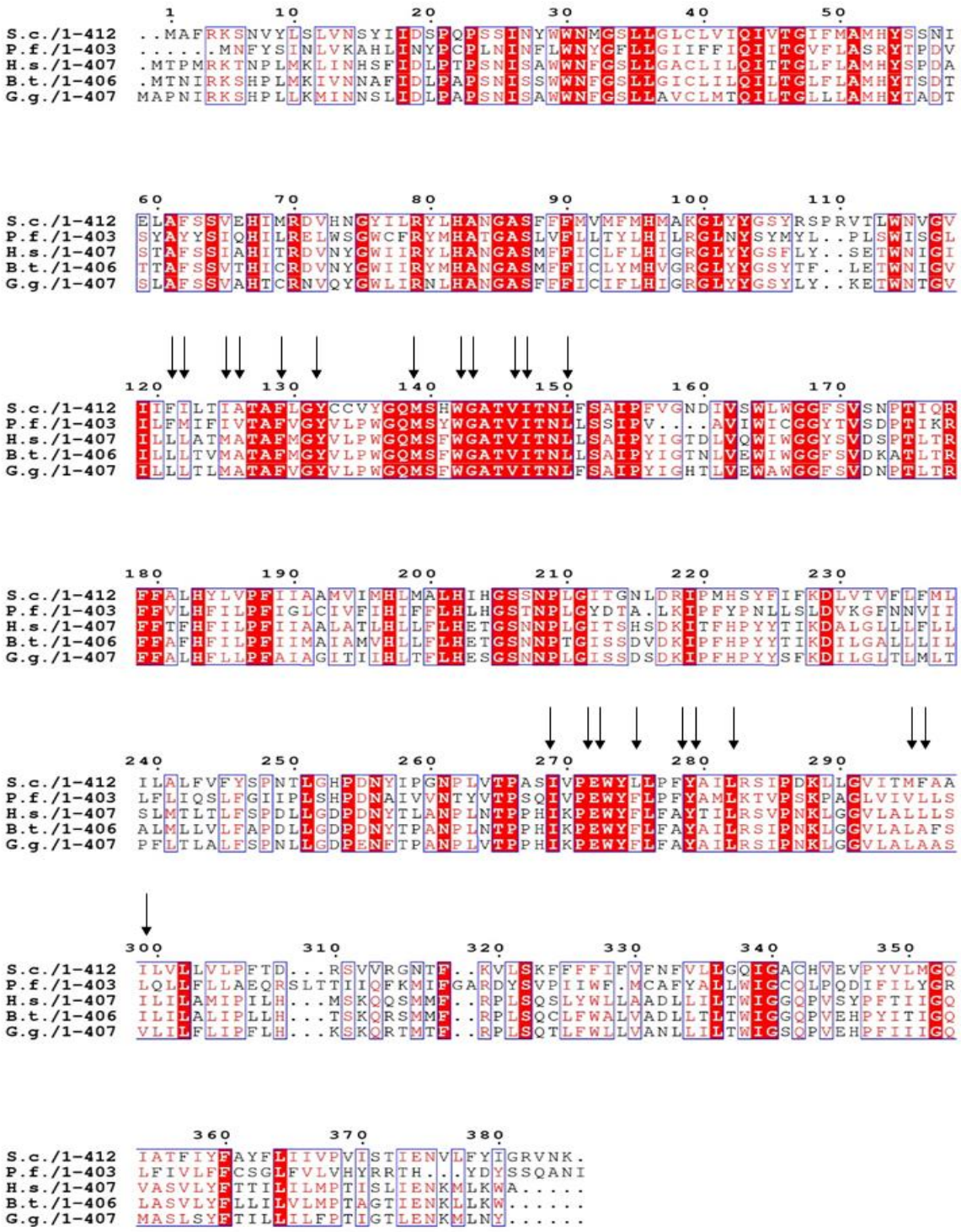

Figure 1. Alignement de séquences du cytochrome *b* de différentes espèces.
S. c. : *Saccharomyces cerevisiae*, P. f.: *Plasmodium falciparum*, H .s.: *Homo sapiens*, B. t.: *Bos Taurus*, G. g.: *Gallus gallus*. Les résidus surlignés en rouge sont des résidus bien conservés parmi ces cinq espèces. Les résidus marqués en rouge sont des résidus qui ont des propriétés physico-chimiques très similaires, donc partiellement conservés. Les rectangles indiquent des résidus bien conservés ou partiellement conservés. Les résidus en noir sont des résidus non conservés. Le point noir dans la séquence représente un décalage d'une séquence par rapport à une autre. Les flèches indiquent les résidus qui, dans la structure du complexe cristallisé avec l'atovaquone lié dans le site Q_o, se trouvent dans une distance inférieure à 5Å de l'inhibiteur. Ce sont aussi des résidus constitutifs du site Q_o.

Étant donné la conservation des séquences du cytochrome *b*, il est possible de construire la structure du cytochrome *b* de *Plasmodium*, par homologie avec la structure du cytochrome *b* de levure disponible (Figure 2). La structure obtenue est très similaire à celle de la levure, surtout pour les deux sites actifs et autour des deux hèmes. Cela suggère que la conservation structurelle du cytochrome *b* pourrait être plus importante que la conservation de séquence.

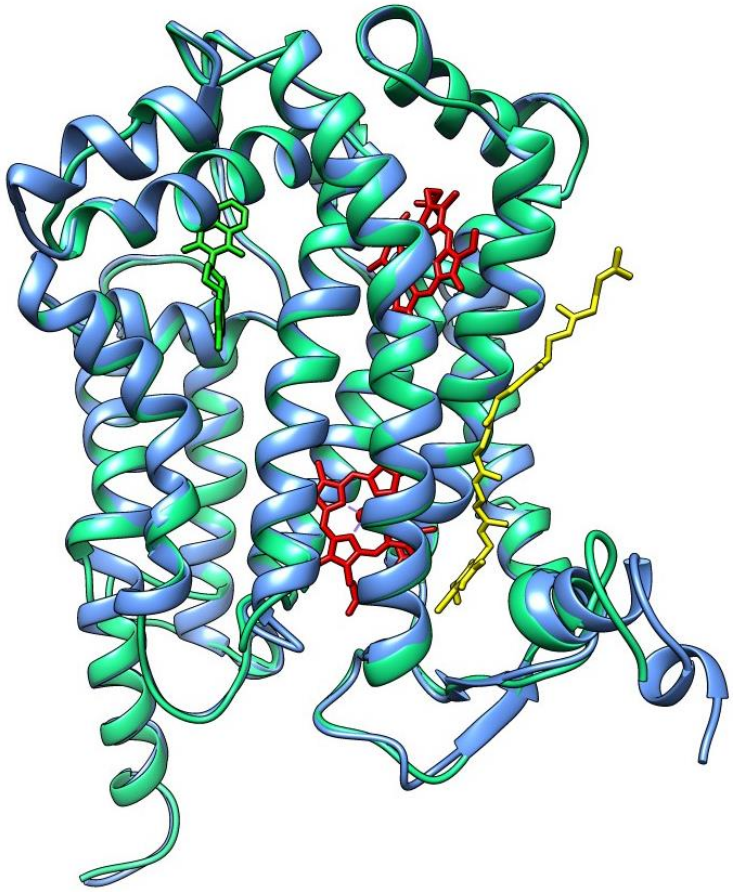

Figure 2. Reconstruction de la structure du cytochrome *b* de Plasmodium (en vert) à partir de la structure cristallographique de la levure (en bleu).
La molécule en vert est l'atovaquone (site Q_o); les deux molécules rouges sont les hèmes b_L et b_H, du haut en bas ; la molécule jaune est l'ubiquinone-6 (site Q_i). La reconstruction est faite par « MODELLER » (Sali and Blundell 1993) et l'image est produite par « Chimera » (Pettersen *et al.* 2004).

B. La mitochondrie, sa structure et fonction

Chez les eucaryotes, la cellule a besoin d'une quantité beaucoup plus importante d'énergie par rapport à une cellule procaryote étant donné sa complexité : complexité morphologique et complexité de sa compartimentation. Pour garantir cette production d'énergie, toutes les cellules eucaryotes nucléées sont équipées d'une organelle, la mitochondrie. Il est maintenant bien accepté que la mitochondrie, comme le chloroplaste, serait le résultat d'une symbiose qui s'est passé il y a environ un milliard d'année, probablement entre une cellule procaryote et une α-protéobactérie (Yang *et al.* 1985) (Gray 1999).

Un des arguments qui soutiennent cette hypothèse de symbiose est que la mitochondrie contient deux membranes lipidiques : la membrane interne et la membrane externe. Dans une cellule, la membrane externe est liée avec la membrane externe des autres mitochondries pour former un réseau. La membrane interne forme beaucoup d'invagination, les crêtes mitochondriales. Entre les deux membranes est l'espace intermembranaire. Cet espace est acide en conditions physiologiques à la suite de l'accumulation de

protons pompés par la chaîne respiratoire située dans la membrane interne. Cette concentration en proton crée un potentiel membranaire qui est à la fois indispensable pour la synthèse d'ATP par le complexe V, et aussi indispensable pour la translocation des protéines composantes de la mitochondrie mais synthétisées dans le cytosol. La translocation de ces protéines et aussi de métabolites, est assurée par des transporteurs situés dans les deux membranes. À l'intérieur de la membrane interne se trouve la matrice mitochondriale.

La mitochondrie est une organelle semi-autonome, parce qu'elle possède son propre ADN contenant seulement une trentaine de gènes, gènes codant pour des protéines très hydrophobes de la chaîne respiratoire et gènes codant pour les ARNs de transfert et ribosomiques mitochondriaux (Figure 3).

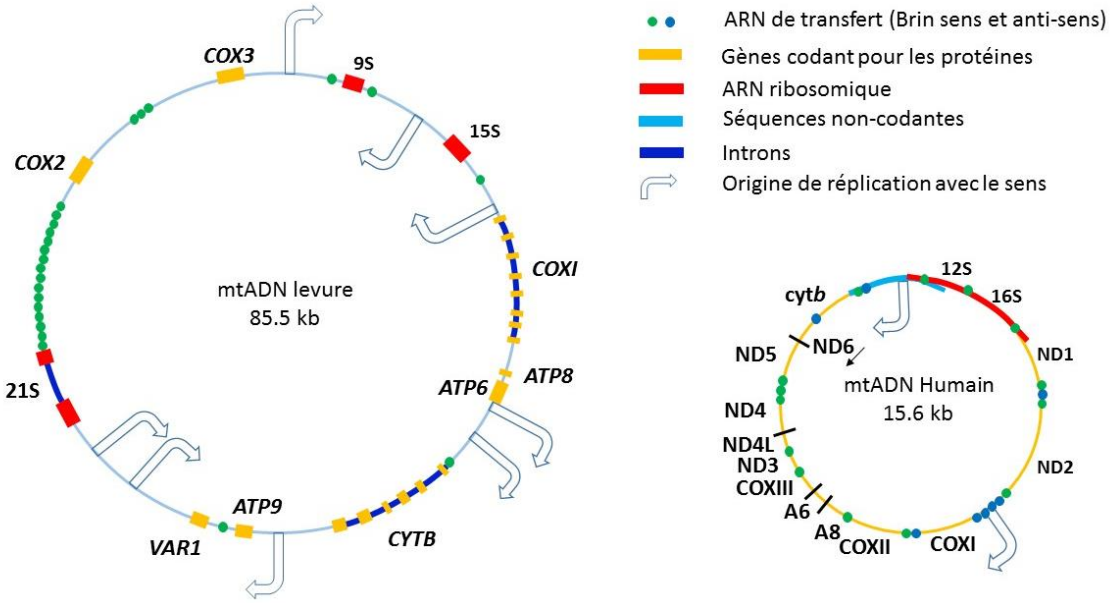

Figure 3. Comparaison des génomes mitochondriaux entre l'homme et la levure.
Les noms des gènes sont en gras. Ceux qui sont à l'extérieur de l'ADN circulaire sont transcrits dans le sens indiqué par la flèche extérieure, ceux qui sont à l'intérieur sont transcrits dans le sens opposé. Cette figure est inspirée de l'article (Jacobs 2001).

D'après l'hypothèse la plus acceptée, pendant l'évolution, la mitochondrie a perdu au fur et à mesure la plupart de ses gènes redondants et/ou a transloqué certains gènes dans le génome nucléaire de la cellule hôte. La mitochondrie a finalement gardé seulement des gènes qui codent pour des protéines essentiels pour la fonction respiratoire et trop hydrophobes pour traverser les deux membranes lipidiques, et aussi les gènes des ARNs de transfert et ribosomiques qui fonctionnent seulement dans la mitochondrie.

L'ADN mitochondrial est, dans la plupart des cellules, circulaire. Contrairement à la cellule humaine, la levure - la plupart des souches de laboratoire - possède beaucoup d'introns dans les gènes mitochondriaux. Pour faciliter la mutagénèse dirigée dans le gène mitochondrial CYTB qui code pour la protéine cytochrome *b*, nous avons utilisé des souches de levure qui n'ont pas d'introns.

Les mitochondries forment un réseau très dynamique dans une cellule. En fonction de l'espèce et du tissu pour un même organisme, le nombre de mitochondries varie, ainsi que le nombre de copies d'ADN mitochondrial. Chez la levure, il y a environ 50 copies d'ADN mitochondrial per cellule.

En plus de la production d'énergie par la chaîne de phosphorylation oxydative, la mitochondrie est aussi essentielle pour accomplir diverses fonctions :

- **Conversion énergétique** : La β-oxydation et le cycle de Krebs transforme l'énergie conservée dans les longues chaînes aliphatiques (acyl-CoA) et de courtes chaînes aliphatiques (acétyl-CoA) en NADH ou $FADH_2$ utilisés ensuite par la chaîne respiratoire pour produire l'ATP.
- **Synthèses** : La synthèse des hèmes et du groupe Fer-Soufre, sont nécessaires pour le fonctionnement de nombreuses protéines dont les cytochromes, la protéine Fer-Soufre (ISP), l'aconitase, etc. Certains stéroïdes sont aussi synthétisés dans la mitochondrie, par exemple, chez l'Homme, la conversion du cholestérol en prégnélonone, une étape de la synthèse de progestogène et d'autres hormones stéroïdes humaines, se passe dans la mitochondrie, ainsi qu'une partie du cycle de l'urée.
  Dans beaucoup d'eucaryotes, la dihydroorotate déshydrogénase, qui est une enzyme essentielle pour la biosynthèse *de novo* des pyrimidines, se localise dans la membrane interne de la mitochondrie. Elle utilise le pool de coenzyme Q comme cofacteur. Elle a donc un lien avec la chaîne respiratoire.
- **Stockage du calcium** : Avec des pompes à calcium et la matrice mitochondriale riche en calcium, la mitochondrie joue ainsi un rôle important dans l'homéostasie du calcium intracellulaire, notamment dans la signalisation liée au calcium et l'apoptose calcium dépendante.

C. La chaîne de la phosphorylation oxydative (OxPhos)

La chaîne de la phosphorylation oxydative se situe dans la membrane interne de la mitochondrie. Elle est composée de la chaîne respiratoire (du complexe I au complexe IV dans la plupart des espèces eucaryotes) et du complexe V ou ATP synthase. Chez la levure, le complexe I - NADH déshydrogénase n'existe pas, des NADH déshydrogénases alternatives localisées à la membrane interne permettent d'oxyder le NADH et de réduire l'ubiquinone (Figure 4). Proposée par Peter. D. Mitchell en 1961 (Mitchell 1961) et bien acceptée actuellement, la théorie chimiosmotique explique que la chaîne respiratoire couple le transfert des électrons au transfert vectoriel des protons à travers la membrane interne mitochondriale, de la matrice vers l'espace intermembranaire, ce qui crée un gradient de protons. Ce gradient est utilisé par le complexe V (ATP synthase) pour produire de l'ATP. Ce gradient électrochimique est aussi nécessaire pour l'entrée des nombreuses protéines résidant dans la mitochondrie et requises pour toutes les fonctions décrites au point B, mais codées par le génome nucléaire et synthétisées dans le cytoplasme (analyses protéomiques montrent qu'il y a environ 800 protéines mitochondriales chez la levure (Sickmann *et al.* 2003) et 1500 chez l'Homme (Taylor *et al.* 2003)).

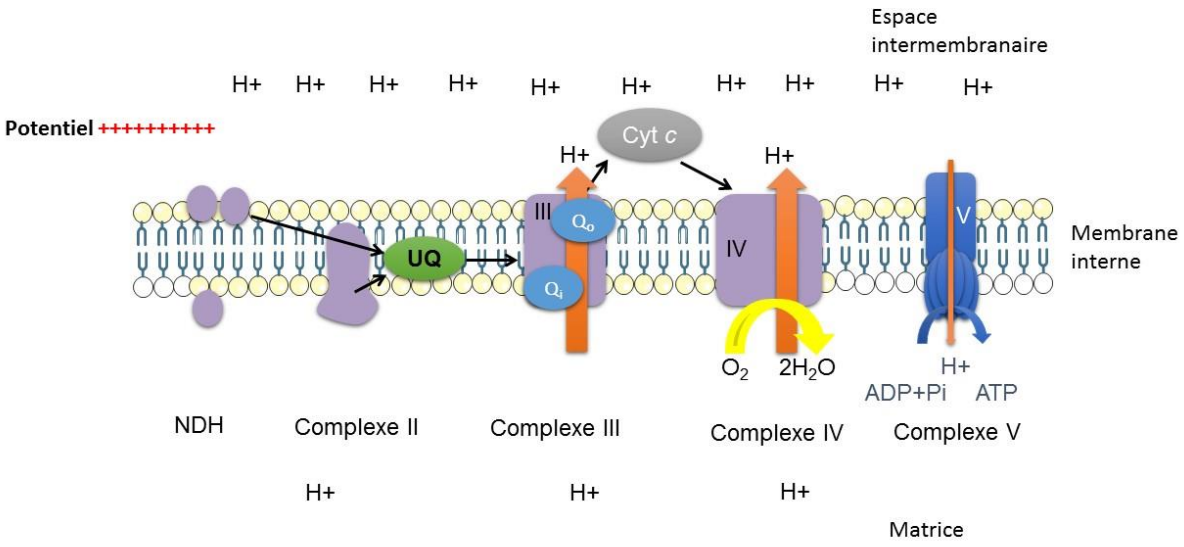

Figure 4. La chaîne de la phosphorylation oxydative.
H^+ : protons qui permet de créer le potentiel membranaire.

D. Complexe *bc*1

Le complexe *bc*1, ou complexe III (Coenzyme Q – cytochrome *c* réductase) est sous forme de dimère. Il est composé de 10-11 sous-unités par monomère, selon l'espèce. Dans chaque monomère, il y a trois sous-unités catalytiques, le cytochrome *b*, le cytochrome c_1 et la protéine Fer-Soufre (Iron Sulphur Protein, ou ISP) (Figure 5). Toutes les sous-unités sont codées par le génome nucléaire, sauf le cytochrome *b*, qui est codé par le génome mitochondrial.

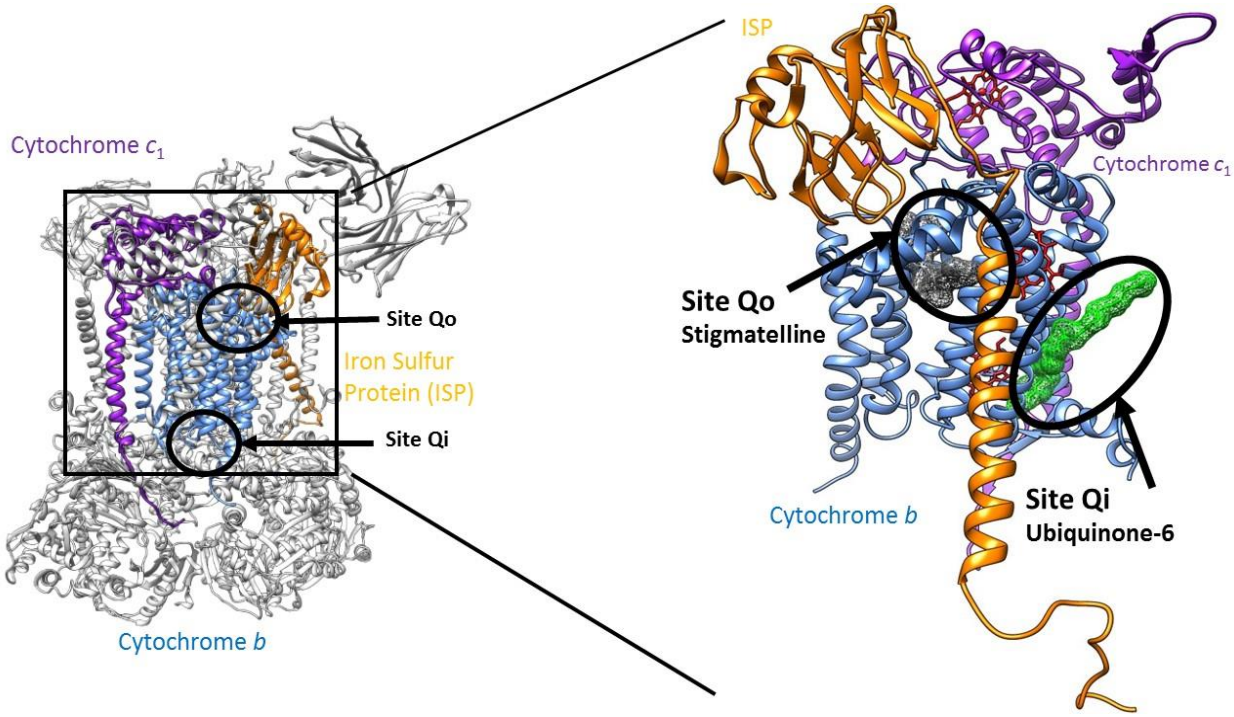

Figure 5. Représentation du complexe bc_1 (gauche) et le cœur catalytique (droite).
Le cytochrome *b* est coloré en bleu, le cytochrome c_1 en violet, la protéine fer-soufre ISP en orange. À droite, le site Q_o et Q_i sont indiqués par la stigmatelline (noire) et l'ubiquinone-6 (verte), respectivement. Les codes PDB des structures utilisées sont 3CX5 et 4PD4.

Beaucoup de structures cristallographiques du complexe bc_1 avec différents inhibiteurs sont disponibles à nos jours (Tableau 2) : huit pour l'enzyme de levure (*Saccharomyces cerevisiae*), 22 pour l'enzyme bovine (*Bos Taurus*) et 17 pour l'enzyme de poulet (*Gallus gallus*), 6 pour l'enzyme des bactéries (4 pour *Rhodobacter sphaeroides*, 1 pour *Rhodobacter capsulatus* et 1 pour *Paracoccus denitrificans*).

Tableau 2. Les structures du complexe bc_1 disponibles au 23 mars 2016.

Code PDB	Résolution (Å)	Espèces	Inhibiteurs et substrat (Q_o)	Inhibiteurs et substrat (Q_i)	Date de publication
3CX5	1.9	*S. c.*	stigmatelline A		2008
3CXH	2.5	*S. c.*	stigmatelline A		2008
1EZV	2.3	*S. c.*	stigmatelline A	Ubiquinone-6	2000
1KB9	2.3	*S. c.*	stigmatelline A	Ubiquinone-6	2001
1KYO	2.97	*S. c.*	stigmatelline A		2002
1P84	2.5	*S. c.*	HDBT	Ubiquinone-6	2003
2IBZ	2.3	*S. c.*	stigmatelline A	Ubiquinone-6	2007
4PD4	3.04	*S. c.*	Atovaquone	Ubiquinone-6	2014
2A06	2.1	*B. t.*	Stigmatelline A	Ubiquinone (Q10)	2005
1PP9	2.1	*B. t.*	Stigmatelline A	Ubiquinone (Q10)	2005
1PPJ	2.1	*B. t.*	Stigmatelline A	Antimycine	2005
1L0L	2.35	*B. t.*	Famoxadone		2003

1L0N	2.6	*B. t.*			2003
1NTK	2.6	*B. t.*		Antimycine A1	2003
1NTM	2.4	*B. t.*			2003
1NTZ	2.6	*B. t.*		Ubiquinone-2	2003
1NU1	3.2	*B. t.*		NQNO	2003
1SQB	2.69	*B. t.*	Azoxystrobine		2004
1SQP	2.7	*B. t.*	Myxothiazole		2004
1SQQ	3	*B. t.*	Methoxy Acrylate Stilbène (MOAS)	Ubiquinone-2	2004
1SQV	2.85	*B. t.*	UHDBT	Ubiquinone-2	2004
1SQX	2.6	*B. t.*	Stigmatelline A	Ubiquinone-2	2004
1BE3	3	*B. t.*			1998
1BGY	3	*B. t.*			1998
4D6T	3.57	*B. t.*		4(1H)-pyridone GW844520	2015
4D6U	4.09	*B. t.*		4(1H)-pyridone GSK932121	2015
2YBB	19	*B. t.*	Stigmatelline A	Ubiquinone-1	2011
1RIE	1.5	*B. t.*			1996
1QCR	2.7	*B. t.*			1997
2FYU	2.26	*B. t.*	jg144(DFN)		2006
3L70	2.75	*G. g*	Trifloxystrobine	Ubiquinone-10	2009
3L71	2.84	*G. g*	Azoxystrobine	Ubiquinone-10	2009
3L72	3.06	*G. g.*	Kresoxym-I-dimethyl	Ubiquinone-10	2009
3L73	3.04	*G. g.*	JZZ (Triazolone)	Ubiquinone-10	2009
3L74	2.76	*G .g*	Famoxadone	Ubiquinone-10	2009
3L75	2.79	*G .g*	Fenamidone	Ubiquinone-10	2009
3H1H	3.16	*G .g.*		Ubiquinone-10	1998
3H1I	3.53	*G .g.*	Stigmatellin	Antimycine	1998
3H1J	3	*G .g.*	Stigmatellin	Ubiquinone-10	1998
3H1K	3.48	*G .g.*	Kresoxym-I-dimethyl	Ubiquinone-10	2000
3H1L	3.21	*G .g.*	Ascochlorin		2010
3CWB	3.51	*G .g.*	Iodinated analogue of the polyketide Crocacine-D	Ubiquinone-10	2008
3TGU	2.7	*G .g.*	pfvs-designed moa	Ubiquinone-10	2012
1BCC	3.16	*G .g.*		Ubiquinone-10	1998
2BCC	3.5	*G .g.*	Stigmatellin	Ubiquinone-10	1998
3BCC	3.7	*G. g.* + *polymère 9=B. t.*	Stigmatellin	Antimycine	1998
4U3F	3.23	*G. g.*	Y52	Ubiquinone-10	2014

Ce complexe contient deux sites catalytiques Q_o et Q_i (Figure 6). Le site Q_i est formé entièrement par le cytochrome *b*. Le site Q_o est formé majoritairement par le cytochrome *b* mais aussi par l'ISP. Ce dernier a une longue hélice hydrophobe qui s'insère dans la membrane. Sa partie globulaire, qui porte le groupe

redox [2Fe-2S] oscille entre le site Q_o et le cytochrome c_1. La structure qui assure la mobilité et le bon positionnement de la partie globulaire s'appelle la région charnière.

Dans le site Q_o, selon l'endroit où se lie le ligand, deux régions se distinguent : le site proximal et le site distal. Le site proximal est proche de l'hème b_L et le site distal est éloigné de l'hème b_L (Figure 6A).

L'ubiquinol, le substrat du complexe bc_1, se lie dans les sites Q_o et Q_i. La structure cristallographique a révélé sa position dans le site Q_i. Par contre, aucune structure n'a permis de déterminer sa localisation dans le site Q_o. Celle-ci est pour l'instant inférée par la position des inhibiteurs distaux comme stigmatelline, atovaquone, HDBT, etc.

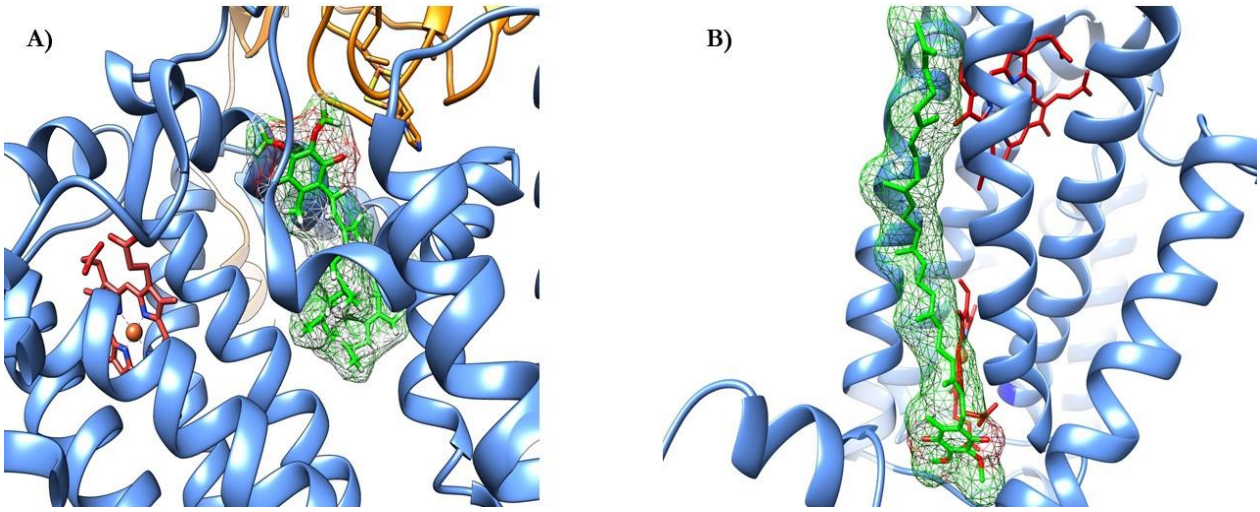

Figure 6. Position de l'ubiquinol-6 dans le site Q_o et le site Q_i.
Le cytochrome *b* est coloré en bleu, l'ISP en orange, les hèmes en rouge ; l'ubiquinol-6, en vert. **A) Site Q_o.** L'ubiquinol-6 se lierait dans la région distale du site Q_o. Son docking a été réalisé en utilisant la structure PDB.3CX5. **B) site Q_i.** La structure PDB.4PD4 contenant l'ubiquinol lié dans le site a été utilisée.

D.1. Le cycle Q

La réaction catalysée par le complexe bc_1 est appelée le cycle Q (Figure 7) (Mitchell 1975) (Trumpower 1976) (Slater 1983) (Crofts 2004).

Le cycle Q est divisé en deux étapes:

Première étape, une molécule d'ubiquinol-6 (chez les animaux, le substrat est l'ubiquinol-10) entre dans le site Q_o. Dans ce site, en position dite distale son groupement benzoquinone hydroxyle se lie à l'H181 de l'ISP via une liaison hydrogène. Un électron est transféré au groupe [2Fe-2S] de l'ISP en même temps qu'un proton est libéré de l'hydroxyle du substrat. La tête globulaire de l'ISP oscillant ensuite vers le cytochrome c_1, l'électron va réduire ce dernier. Une fois l'électron transféré au cytochrome c_1, la tête globulaire de l'ISP retourne vers le site Q_o du cytochrome *b*. L'ubiquinol qui a perdu un hydrogène et un électron est temporairement sous forme de semiquinone intermédiaire, très réactif. Cette semiquinone bougerait alors vers le site proximal pour libérer son hydrogène du groupement benzoquinone hydroxyle en position - para à l'acide glutamique 272 et pour transférer un deuxième électron à l'hème b_L. L'ubiquinol est ainsi oxydé en ubiquinone et quitte le site Q_o. L'électron est ensuite passé de l'hème b_L à

l'hème b_H qui se situe dans le site Q_i. Cet électron est finalement transféré à la molécule ubiquinone-6 pour créer une semiquinone plus fortement liée et plus stable que celle du site Q_o.

Les formules des réactions individuelles pour la première partie du cycle Q dans les deux sites sont

(EI, espace intermembranaire ; QH_2 ubiquinol ; Q, quinone ; QH•° semiquinol):

Site Q_o : $QH_2 \rightarrow Q + 2H^+(EI) + 2e^-$

Site Q_i : $Q + H^+$ (matrice) $+ e^- \rightarrow QH•$

→La formule de la réaction globale pour la première partie du cycle Q est (EI pour espace intermembranaire):

QH_2 (Q_o) + H^+ (matrice) → QH• (Q_i) + $2H^+$ (EI) + e^-

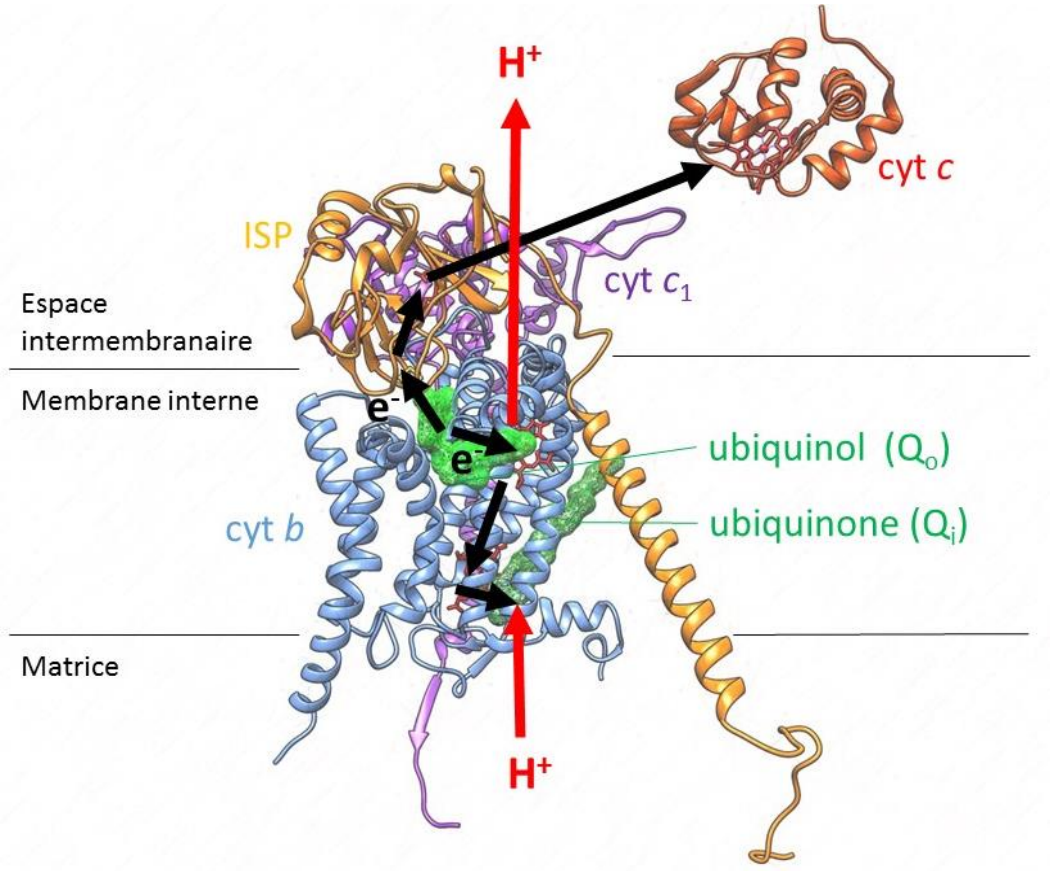

Figure 7. Représentation simplifiée du cycle Q.
Le cytochrome *b* est coloré en bleu, l'ISP en orange, le cytochrome c_1 en violet, le cytochrome *c* en rouge et les substrats en vert. Le sens du flux d'électrons et du flux de protons est indiqué par les flèches noires et rouges, respectivement.

Deuxième étape, une nouvelle molécule d'ubiquinol entre dans le site Q_o. La même réaction a lieu. L'électron transféré au site Q_i réduit alors la semiquinone en ubiquinol, qui quitte le site Q_i. Les formules des réactions individuelles pour la deuxième partie dans les deux sites sont :

Site Q_o : $QH_2 \rightarrow Q + 2H^+(EI) + 2e^-$

Site Q_i : $QH• + H^+$ (matrice) $+ e^- \rightarrow QH_2$

→La formule de la réaction globale pour la deuxième partie du cycle Q est (EI pour espace intermembranaire):

$QH\bullet(Q_i) + H^+$ (matrice) → Q (Q_o) + $2H^+$(EI) + e^-

→La réaction globale d'un cycle Q est :

$QH_2 + 2H^+$ (matrice) → $Q + 4H^+$(EI) + $2e^-$

Les deux électrons libérés dans le site Q_o successivement durant chaque cycle réduisent finalement deux molécules de cytochrome c_1 (Fe^{3+}) oxydés en deux cytochromes c_1 (Fe^{2+}) réduits. Les deux cytochromes c_1 réduits réduisent ensuite deux cytochrome c, sous-unité mobile qui permet de transporter les électrons entre le complexe bc_1 et le complexe IV.

Donc durant chaque cycle complet, une molécule d'ubiquinol est oxydée en ubiquinone, deux cytochromes c_1 sont réduits avec le mouvement concomitant de six protons de la matrice vers l'espace intermembranaire.

D.2. Production des espèces réactives d'oxygène (ROS) par le complexe bc_1

Les espèces réactives d'oxygène (ROS) sont formées naturellement dans la cellule. Les ROS ont un rôle important dans la signalisation cellulaire. Mais elles sont plus connues pour les dégâts qu'elles peuvent induire dans la cellule quand elles sont en trop grande quantité.

Il y a deux « hotspots » de production de ROS. L'un est NADPH oxydase (NOX) (Block and Gorin 2012) qui est un producteur de ROS dit « professionnel » et se situe sur la membrane cellulaire et aussi sur les membranes de certains compartiments, tel que le peroxysome, le réticulum endoplasmique, etc. L'autre est la chaîne respiratoire dans la mitochondrie, plus précisément les complexes I et bc_1 (X. Li *et al.* 2013) (Dröse and Brandt 2012). La levure *S. cerevisiae* a une protéine Yno1p de la superfamille NOX qui se localise sur le réticulum endoplasmique péri-nucléaire, cette protéine étant capable de produire de ROS (Rinnerthaler *et al.* 2012). La levure n'a pas de complexe I, mais son NADH déshydrogénase externe mitochondriale, qui remplit partiellement la fonction du complexe I, a été montrée comme une source potentielle de la production de ROS (Fang and Beattie 2003).

Comme décrit dans « D.1 Le cycle Q », lors de l'oxydation de l'ubiquinol dans le site Q_o, une semiquinone intermédiaire est produite. Cette semiquinone est très réactive et réagit avec l'oxygène pour produire des ROS (plus spécifiquement des ions superoxide, ou SO). Lors du cycle catalytique en conditions normales, la durée de la présence de cette semiquinone intermédiaire est très courte et sa concentration donc très limitée, ce qui fait que le complexe bc_1 produit peu de ROS. Mais des mutations du site Q_o ou d'autres modifications peuvent augmenter sa concentration dans le site Q_o, ce qui résulte alors en surproduction de ROS.

E. Le paludisme et les médicaments

E.1. Le paludisme

L'Organisation Mondiale de la Santé estime qu'en 2015, il y avait 214 million de cas de paludisme et environ 438,000 personnes sont mortes à cause de cette maladie infectieuse (WHO 2015). Le paludisme, ou la malaria, est causé par un parasite protozoaire apicomplexe, du genre *Plasmodium*. Il est transmis par les moustiques anophèles. Cinq espèces de *Plasmodium* peuvent infecter l'Homme : *P. falciparum, P. vivax, P. ovale, P. malariae* et *P. knowlesi*. Parmi eux, *P. falciparum* et *P. vivax* sont les plus répandus. Le cycle de réplication du *Plasmodium* est complexe. Une partie se déroule chez le moustique et une autre chez l'Homme. Les symptômes peuvent aller de la fièvre et tremblement intermittents jusqu'à l'anémie, l'ictère et la convulsion, voire le décès. *P. falciparum* est responsable du symptôme le plus sévère et de la grande majorité de décès suite à une infection. Chez les enfants infectés, il pourrait causer le neuro-paludisme avec une atteinte des parasites dans le tissu nerveux central. L'enfant présentant ce symptôme a un grand risque de développer des dommages irréparables dans le cerveau, voire de mourir. *P. vivax, P. ovale et P. malariae* ont une particularité de produire des parasites sous « formes dormantes », ou des hypnozoïtes, durant leur cycle de réplication chez l'Homme. Ces hypnozoïtes peuvent survivre longtemps dans le foie de l'hôte sans causer de symptôme. Mais ils peuvent être réactivés dans certaines conditions et causer le Paludisme *de novo*.

E.2. L'atovaquone, un médicament antipaludique

Le médicament commercialisé sous le nom de « Malarone® » en 2000 par GlaxoSmithKline est composé d'atovaquone et de proguanil. L'atovaquone est un inhibiteur du site Q_o du complexe bc_1 du parasite. Elle bloque le transfert d'électron de la chaîne respiratoire et ainsi diminue le potentiel membranaire de la mitochondrie parasitaire. L'activité du complexe bc_1 étant inhibé, la dihydroorotate déshydrogénase, essentielle pour la biosynthèse *de novo* des pyrimidines (Gutteridge 1979), s'arrête également. En effet, chez le parasite, l'activité catalytique de cette enzyme requiert la ré-oxydation de l'ubiquinol par le complexe bc_1.
Le proguanil est un biguanide qui diminue la concentration effective de l'atovaquone requise pour le collapse du potentiel membranaire mitochondrial (Srivastava and Vaidya 1999). Une fois métabolisée en cycloguanil, la molécule devient un inhibiteur de la dihydrofolate réductase (DHFR) et donc perturbe la synthèse des purines, des pyrimidines et des acides aminés chez *Plasmodium falciparum* (Hyde 2005). Ces deux molécules ont un effet synergique résultant dans une déficience en purine et pyrimidines, ce qui compromet la réplication et la survie du pathogène.

L'atovaquone est un hydroxy-1,4-naphtoquinone, analogue d'ubiquinone. Il a une très bonne affinité pour le complexe bc_1 des pathogènes, tels que le *Plasmodium sp.* , *Pneumocystis sp.* et *Toxoplasma gondii* (Siregar *et al.* 2015) (Kessl *et al.* 2004) (Kessl *et al.* 2006). Le complexe bc_1 semble une bonne cible thérapeutique. Cependant, l'atovaquone est actuellement le seul médicament antipaludique ciblant le complexe bc_1 sur le marché. Malheureusement, des échecs cliniques causés par des mutations de résistance sont reportés régulièrement. Des mutations dans le cytochrome *b* parasitaire, surtout à la position 279, comme Y279S, Y279C ou Y279N, sont souvent observées dans les cas de résistance. Donc pour contourner ce problème

de résistance au site Q_o, et en même temps continuer à utiliser le complexe bc_1 comme cible thérapeutique, plusieurs équipes sont en train de développer d'autres molécules. L'équipe de M. Riscoe, aux États-Unis, a produit et analysé une série de molécules « Endochin-like Quinolones » qui pourraient cibler le site Q_i. Nous avons testé certaines de ces molécules avec notre modèle levure, décrit dans le « Chapitre-IIA ». D'autres équipes se focalisent sur la modification de l'efficacité de l'atovaquone, soit en le rendant plus efficace pour inhiber le complexe bc_1 parasitaire en l'administrant avec une émulsion spéciale, soit en modifiant des groupements de la structure de base pour essayer d'augmenter son affinité au complexe mutant.

De nombreux autres médicaments antipaludiques qui ciblent d'autres fonctions du parasite existent. Par exemple, la quinine et la chloroquine qui inhibent la biocrystallisation de l'hémozoïne, un processus indispensable au parasite pour neutraliser l'hème cytotoxique ; Artémisine et ses dérivés, et la primaquine, qui n'ont pas de cible bien identifiée. Elles agissent probablement via un stress oxydant.

Introduction aux problématiques

Introduction aux problématiques de cette thèse

Je vais présenter les problématiques en fonction de la localisation des résidus étudiés, c'est-à-dire site Q_o ou site Q_i.

Parce qu'il y a une variation de la séquence du cytochrome *b* entre les différentes espèces, la numérotation des résidus correspondants est différente dans chaque espèce. Dans ce travail, je vais utiliser la numérotation de la levure sauf indication contraire.

A. Site Q_o

A.1. Inhibiteurs du site Q_o

Le site Q_o peut être divisé en deux parties : le site proximal à l'hème b_L et le site distal où se lierait initialement le substrat ubiquinol. Le mécanisme catalytique du complexe bc_1 est décrit dans l'Introduction Générale point « D.1 ». Je rappelle ici qu'après le transfert du premier électron, l'ubiquinol initialement au site distal est transformé en une semiquinone, très réactive. Cette semiquinone tournerait pour entrer dans le site proximal et pour transférer le deuxième électron à l'hème b_L (Figure 8).

Il faut noter qu'il n'existe pas de structure atomique du complexe bc_1 avec le substrat lié au site Q_o. Sa localisation dans la structure est inférée par la liaison d'inhibiteurs ainsi que de l'analyse de mutants de ce site.

De nombreux inhibiteurs ciblant le site Q_o existent, par exemple : la stigmatelline, l'atovaquone, l'azoxystrobine, le myxothiazole, le famoxadone, la strobilurine, le 5-undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT). Parmi eux, myxothiazole, azoxystrobine, famoxadone et strobilurine se lient dans le site proximal tandis que stigmatelline, atovaquone et UHDBT se lient dans le site distal. Les inhibiteurs du site proximal peuvent générer une surproduction de ROS parce ce qu'ils laissent le site distal disponible pour l'entrée du substrat, mais ils coupent l'accès du deuxième électron de la semiquinone vers l'hème b_L.

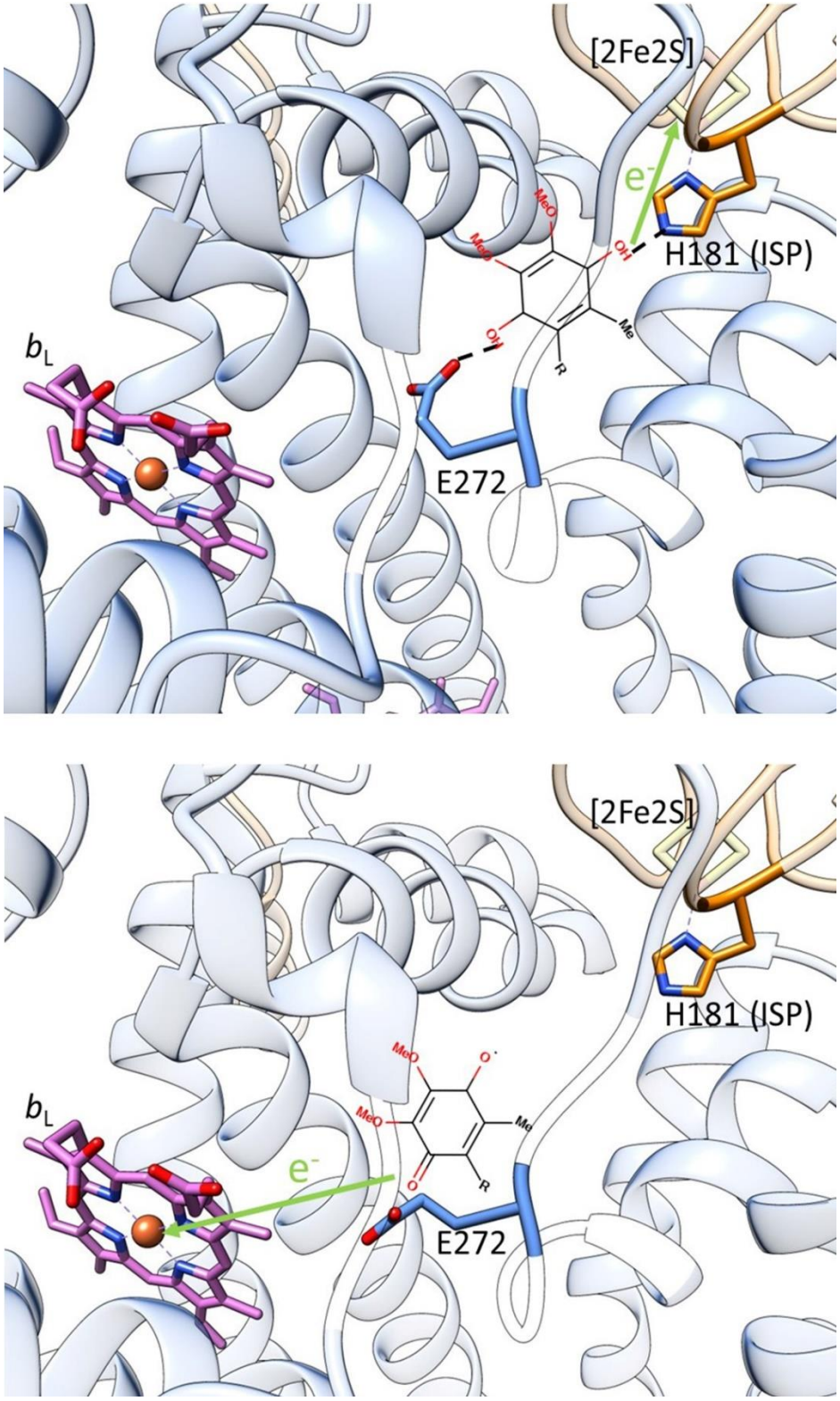

Figure 8. Changement de position de l'ubiquinol dans le site Q_o au cours de la réaction.
(Fisher, Bowman, and Kramer 2016) En haut, les liaisons hydrogène que le substrat peut établir avec H181 de l'ISP et E272 du cytochrome *b* sont indiquées par les lignes discontinues. En bas, changement de localisation de la semiquinone pour transférer son deuxième électron à l'hème b_L. E272 (bleu), l'hème b_L (violet) de cytochrome *b* (ruban) et H181 (orange) de l'ISP sont indiqués. Le substrat est indiqué en formule brute, dont « Me » est un groupement méthyle et R représente la chaîne aliphatique du substrat. Le transfert d'électron est montré avec les flèches vertes.

A.2. Résidus importants dans la liaison du substrat et des inhibiteurs.

Trois résidus du site Q_o ont un rôle clé dans la liaison du substrat et des inhibiteurs : E272 et Y279 du cytochrome *b* et H181 de l'ISP. Ces trois résidus sont très conservés et importants pour l'activité du complexe.

E272 est impliqué dans le mouvement des protons dans le site Q_o. Sa chaîne latérale est très mobile. Elle peut osciller entre l'hème b_L et la semiquinone dans le site Q_o (Wenz *et al.* 2006). H181 se situe dans la tête globulaire de l'ISP. Le résidu participe au maintien du groupe Fer-Soufre et reçoit le premier élection et proton libéré du substrat (Birth, Kao, and Hunte 2014). Y279 serait requise pour une orientation correcte de l'ubiquinol se liant au site Q_o (Palsdottir *et al.* 2003).

L'analyse de différentes structures atomiques a montré que azoxystrobine et famoxadone forment une liaison hydrogène avec le nitrogène du backbone de E272. Stigmatelline et atovaquone forment une liaison hydrogène avec H181 de l'ISP et une liaison hydrogène avec E272. Quand la stigmatelline est liée au site Q_o, Y279 forme une liaison hydrogène avec un oxygène de l'ISP, tandis que cette liaison hydrogène ne peut pas se former pour les trois autres inhibiteurs (Figure 9).

L'atovaquone, avec son groupement hydroxyle ionisé, forme une liaison hydrogène avec H181 et est lié avec le nitrogène du backbone d'E272 par des liaisons hydrogène via une molécule d'eau.

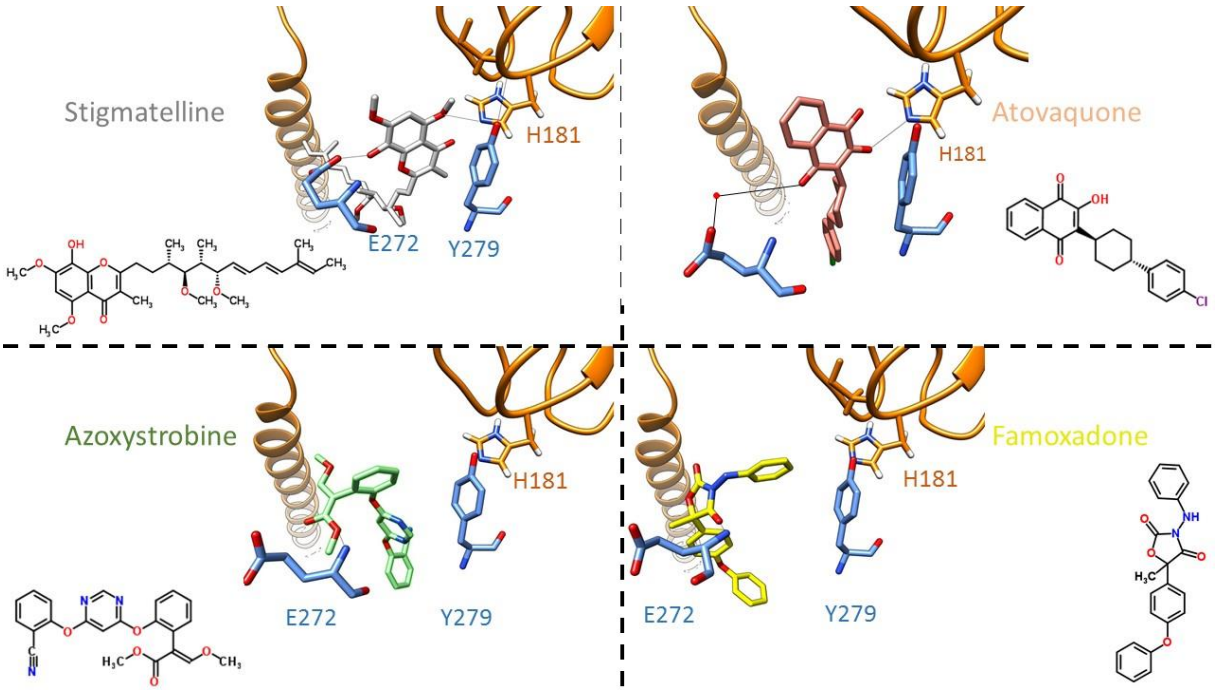

Figure 9. Représentation du positionnement spatial et des liaisons hydrogènes des inhibiteurs du site Q_o.
Stigmatelline (PDB.3CX5) est en gris, atovaquone (PDB.4PD4) en saumon, azoxystrobine (PDB.1SQB) en vert clair et famoxadone (PDB.1L0L) en jaune. La protéine ISP est en orange, les deux résidus importants E272 et Y279 du cytochrome *b* sont en bleu. Le point rouge représente une molécule d'eau. Les formules brutes de chaque inhibiteur sont montrées à côté.

La première structure du complexe bc_1 de levure co-cristallisé avec l'atovaquone est disponible depuis 2014 (Birth, Kao, and Hunte 2014). Son analyse permet de comprendre comment l'atovaquone se lie

dans le site Q_o, pourquoi cette molécule a un spectre d'action assez large et comment les mutations de résistance la rendent inefficace.

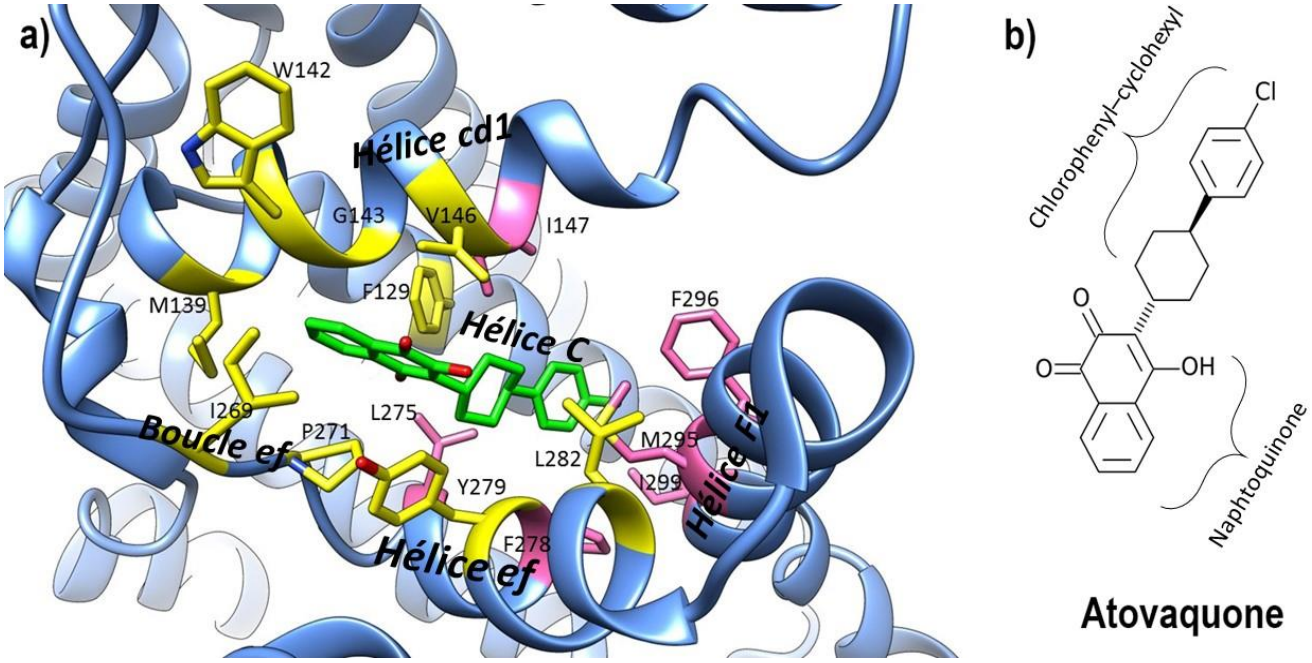

Figure 10. La localisation des résidus qui interagissent avec l'atovaquone a) et l'atovaquone b).
(Birth, Kao, and Hunte 2014) Le cytochrome *b* est coloré en bleu, l'atovaquone en vert, les résidus en contact avec la naphthoquinone (la tête) de l'atovaquone en jaune, et les résidus en contact avec la partie chlorophenyl–cyclohexyl (la queue) en rose.

Comme le montre la Figure 10, le groupement de la tête (naphthoquinone) de l'atovaquone est en contact avec F129, M139, W142, G143, V146, I269, P271, L275, Y279 et L282 du cytochrome *b*. Le groupement de la queue (chlorophenyl–cyclohexyl) de l'atovaquone est en contact avec I147, L275, F278, M295, F296 et I299 du cytochrome *b*. Parmi ces résidus, quatre sont particulièrement importants pour stabiliser l'atovaquone : Y279, V146, G143 et P271. Chez *P. falciparum*, la majorité des cas de résistance à l'atovaquone sont dues à des mutations du résidu Y279. La chaîne latérale aromatique d'Y279 positionne le plan du cycle naphthoquinone de l'atovaquone avec un angle d'environ 59° par rapport à la queue (Birth, Kao, and Hunte 2014). Ceci permettrait de bien positionner l'inhibiteur dans le site actif et de le rendre moins mobile en introduisant une contrainte stérique par la chaîne phénolique. Le bon positionnement permettant de multiples faibles interactions formées avec les résidus en contact résulterait en une liaison assez forte de l'atovaquone dans le site Q_o du parasite. Les mutations d'Y279 qui rendent le positionnement d'atovaquone dans le site Q_o moins précis et stable, diminueraient considérablement l'affinité de l'inhibiteur pour le site muté.

Tableau 3. Niveau de conservation de chaque résidu en contact avec l'atovaquone d'après (Kao and Hunte 2014).

Résidus en contact avec l'atovaquone	Conservation en pourcentage
Contact avec la tête de l'atovaquone	
M139	99
G143	91
P271	100
I269	98
W142	99
F129	99
L275	96
V146	99
Y279	98
L282	99
contact avec la queue de l'atovaquone	
I147	98
M295	86
F278	89
I299	81
F296	45

La conservation des résidus a été analysée en comparant 3556 séquences du cytochrome *b*. Les résidus en gras sont des résidus qui contactent l'atovaquone avec leur backbone.

Les résidus en contact avec l'atovaquone sont très conservés (Tableau 3), surtout V146, P271 et Y279, trois des quatre résidus clés qui stabilisent atovaquone. Ceci expliquerait pourquoi l'atovaquone a un spectre d'action assez large (Kao and Hunte 2014) .

Les quelques différences entre les espèces pourraient expliquer son affinité différentielle chez les pathogènes et chez l'Homme. Notre équipe a montré avant qu'en introduisant 3-8 mutations dans le site Q_o de la levure pour mimer le site Q_o de l'enzyme humaine, sa sensibilité à l'atovaquone a considérablement diminué (Vallières, Fisher, and Meunier 2013). Cela montre que la liaison des inhibiteurs dans le site Q_o est finement coordonnée par les résidus qui forment le site et une légère différence en composition de résidus peut altérer l'affinité du site pour des ligands.

A.3. Sorties de protons

Lors de l'oxydation de l'ubiquinol, deux protons sont transférés vers l'espace intermembranaire (Figure 8). Le premier proton quitte le site Q_o via H181 de la tête globulaire mobile de l'ISP. Le deuxième proton passerait par un réseau de résidus polaires, dont E272, et molécules d'eau. Le mouvement du résidu E272 jouerait un rôle important (Hunte, Palsdottir, and Trumpower 2003).

L'analyse des structures cristallographiques contenant la stigmatelline (Hunte *et al.* 2000) et UHDBT (Palsdottir *et al.* 2003) dans le site Q_o montre que ces inhibiteurs peuvent former une liaison hydrogène avec le groupement imidazole de l'H181 de l'ISP. Cette liaison hydrogène est probablement aussi présente

dans l'interaction du substrat et l'ISP. Elle serait responsable du transfert du proton et de l'électron du groupement hydroxyle du substrat vers l'ISP. Une fois le groupe [2Fe-2S] de l'ISP réduit, la tête mobile migre vers le cytochrome c_1 et passe son électron. Quand l'électron est libéré, le proton est aussi libéré dans l'espace intermembranaire.

La stigmatelline, avec son groupement hydroxyle, peut aussi former une liaison hydrogène avec le groupement carboxylate d'E272 comme le substrat. Lors du cycle catalytique (cycle Q), une fois le second électron transféré à l'hème b_L, cette liaison serait rompue et le proton serait transféré à E272. E272 ainsi protoné ferait une rotation de 170° pour donner le proton à une molécule d'eau qui est liée par une liaison hydrogène avec le propionate de la structure porphyrine de l'hème b_L (Hunte, Palsdottir, and Trumpower 2003). Le proton serait ensuite transféré dans l'espace intermembranaire via une chaîne de molécules d'eau stabilisée par l'hème b_L et les résidus H253, R79, Q256, E66 and R70 successivement du cytochrome *b* (Hunte *et al.* 2000) (Palsdottir *et al.* 2003).

B. Site Q_i

Localisé à l'autre côté de la membrane interne de la mitochondrie, le site Q_i est le second site de liaison du substrat. L'ubiquinone y est réduite en ubiquinol en recevant les électrons transférés par l'hème b_H et les protons provenant de la matrice. Ce site est plus volumineux que le site Q_o.

Comme il a été mentionné auparavant, ce site est particulièrement intéressant dans la recherche des molécules antipaludiques. En effet, l'atovaquone cible le site Q_o et les mutations de résistance Y279S/C/N dans ce site apparaissent fréquemment chez le parasite. Quand un antipaludique ciblant le site Q_i était administré en combinaison avec l'atovaquone, en bithérapie, la possibilité de développer des mutations de résistance dans le deux sites simultanément serait beaucoup plus faible, donc le traitement pourrait être plus efficace (Allison M. Stickles *et al.* 2016).

En plus, il y a une grande différence dans la séquence du site Q_i entre *Plasmodium* et les autres espèces. Le site Q_i du parasite serait donc structurellement moins conservé par rapport au site Q_i d'autres espèces. Cette différence structurelle expliquerait la plus faible sensibilité de *Plasmodium* à l'antimycine, une drogue qui bloque efficacement le site Q_i du complexe bc_1 des bactéries, de la levure et des cellules des mammifères (H. Li *et al.* 2014) (Smilkstein *et al.* 2008).

Le site Q_i du *Plasmodium* est donc une cible intéressante pour le développement des nouveaux antipaludiques (Lukens *et al.* 2015). C'est pour cela que l'équipe de M. Riscoe (Portland, USA) étudie une série de molécules quinolone qui ressemblent à des endochins (ELQ, endochin-like quinolone, Figure 12) (A. Nilsen *et al.* 2013) (J. S. Doggett *et al.* 2012) et une équipe de la compagnie pharmaceutique GlaxoSmithKline étudie des 4(1H)-pyridones. Ils ont montré que certaines de ces molécules se lient dans le site Q_i telles que ELQ-271 et ELQ-300, les 4(1H)-pyridones GSK932121 et GW844520 (Figure 12) (Allison M. Stickles, de Almeida, *et al.* 2015) (Capper *et al.* 2015).

B.1. Inhibiteurs du site Q_i

L'antimycine est une drogue universelle qui bloque efficacement le site Q_i des bactéries, de la levure et des cellules des mammifères. Mais elle est moins efficace chez le *Plasmodium*.

HDQ est un inhibiteur du $PfNDH_2$ (*Plasmodium falciparum* NADH déshydrogénase de type II) et du complexe bc_1 du parasite et de la levure. Son IC_{50} pour le complexe bc_1 est du même ordre de grandeur que l'atovaquone.

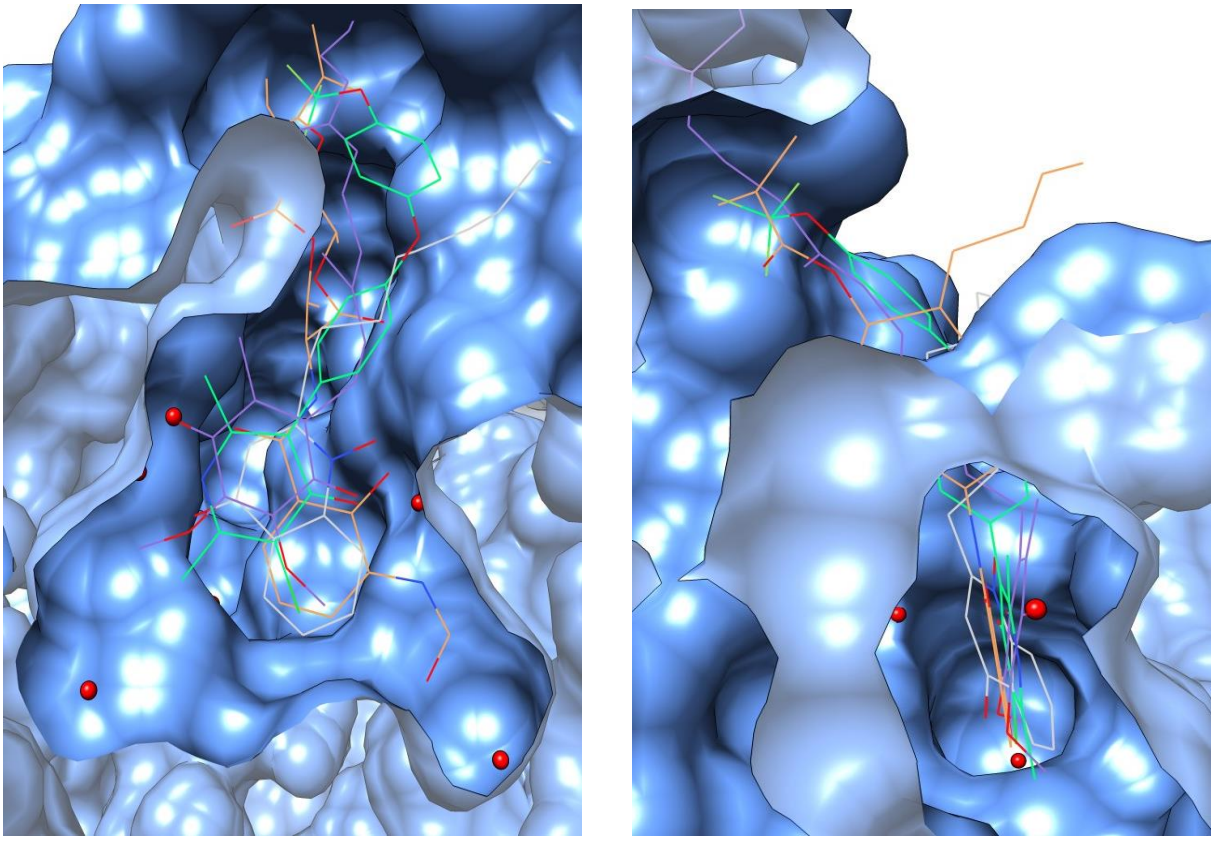

Figure 11. La pose de NQNO (gris), de l'antimycine (saumon), de GSK932121 (vert) et de l'ubiquinone-6 (violet) dans le site Q_i.
Le cytochrome *b* est coloré en bleu. Gauche : vue du dessus du plan d'alignement des cycles aromatiques des molécules. Droite : Vue de côté des molécules. Structure PDB utilisées : 1NU1(NQNO), 1PPJ (antimycine), 4D6T (GSK932121) et 1EZV (ubiquinone-6).

NQNO peut se lier dans les deux sites Q_o et Q_i, mais son affinité pour le site Q_i est plus élevée et il est considéré généralement comme un inhibiteur du site Q_i. Des études de mutations de résistance ont conclu que NQNO se lie différemment que l'antimycine dans le site Q_i (Gao *et al.* 2003).

ELQ (Endochin-like quinolone) est le nom d'une série de molécules synthétisées par l'équipe de M. Riscoe (Portland, USA). Ces molécules ont un effet antipaludique et ciblent le complexe bc_1 (A. Nilsen *et al.* 2014) (R. W. Winter *et al.* 2008). Parmi elles, ELQ-271 est un inhibiteur du parasite plus puissant que l'atovaquone. ELQ-300 est environ 10 fois moins puissant que ce dernier. Mais, ELQ-300 a une faible cytotoxicité contre les cellules mammifères, ce qui le rend un bon candidat pour l'étude préclinique (A. Nilsen *et al.* 2013) (Capper *et al.* 2015) .

Les 4(1H)-pyridones ont été étudiées depuis les années 1960s. Ces molécules ont une activité antipaludique. Les molécules GSK932121 et GW844520 ont été étudiées plus en détail par GlaxoSmithKline. Elles sont des bons inhibiteurs du complexe bc_1 du *Plasmodium*, mais elles ont provoqué une cardiotoxicité aigue chez les souris et une cytotoxicité dans les cellules mammifères, ce qui a entrainé l'arrêt de leur développement en médicaments (Bueno *et al.* 2012) (Xiang *et al.* 2006).

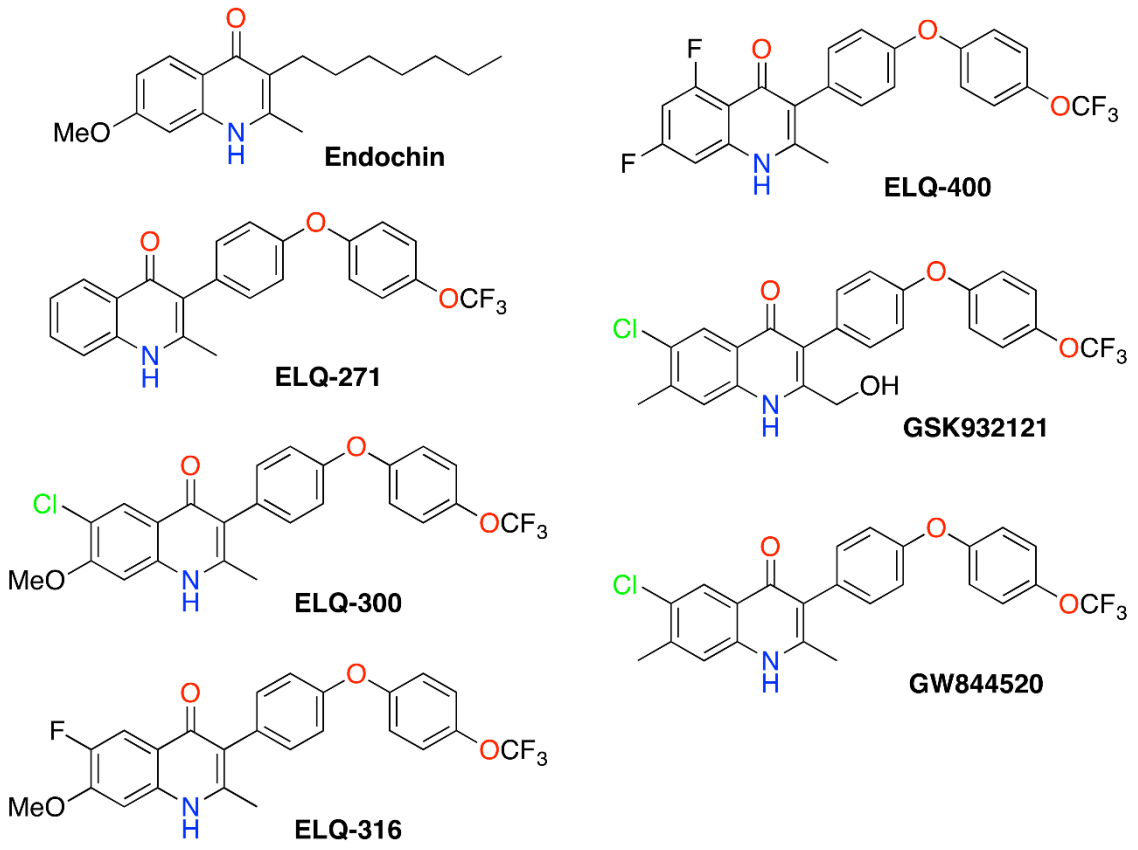

Figure 12. Endochin, Endochin-like quinolones (ELQ) et 4(1H)-pyridones.
GSK932121 et GW844520 sont des 4(1H)-pyridones.

Malgré l'entrée large et le volume spacieux du site Q_i, il y a un point en commun dans la liaison de ces inhibiteurs. Tous ces inhibiteurs ont une extrémité aromatique. Cette extrémité aromatique a vraisemblablement une affinité pour la poche profonde du site Q_i. La comparaison des structures montre que les poses de l'extrémité aromatique de ces inhibiteurs dans le site Q_i sont similaires (Figure 11.). Elles se superposent et sont plus ou moins alignées sur un même plan dans le site Q_i de la levure et du bœuf. Dans le cas d'une mode de liaison qui ne nécessite pas une superposition des cycles aromatiques, ces derniers pourraient se tourner dans le site actif et donc n'auraient pas un plan d'alignement. Ils auraient, entre eux, des angles beaucoup plus grands que ce qui est montré dans la Figure 11.

B.1. Résidus clé pour la fonction

Parmi les 35 résidus dans un rayon de 5Å du substrat ubiquinone lié au site Q_i, 12 résidus interagissant avec la tête aromatiques du substrat semblent les plus importants pour l'activité. Ce sont I17, S20, Q22, I26, W30, S34, L198, L201, G205, M221, F225 et D229. Parmi eux, plusieurs résidus ont une chaîne latérale polaire, tels que S20, W30, S34 et D229, ce qui suggère une interaction avec le groupement carbonyle de l'ubiquinone ou avec une molécule d'eau. D'autres résidus ont une longue chaîne latérale, tels que Q22, W30, M221 et F225, ce qui suggère un rôle dans la stabilisation et/ou l'orientation du substrat dans le site Q_i nécessaire pour une bonne activité.

Les autres résidus formant le site Q_i sont en contact avec la queue aliphatique du substrat. Ces résidus non-chargés apolaires garantissent l'interaction hydrophobe-hydrophobe.

Il faut noter que parmi les 12 résidus en interaction avec la tête aromatique de l'ubiquinol et probablement requis pour un bon fonctionnement, seulement 5 sont bien conservés entre la levure et *Plasmodium* : I26, W30, L201, G205 et D229. Donc, il est possible que chez le parasite, l'interaction entre le site Q_i et l'ubiquinone soit différente de ce que nous observons dans la structure cristallographique de l'enzyme de levure. Comme le montre la Figure 13, la forme du site Q_i semble différer entre la levure et *Plasmodium*. En comparant la structure cristallographique du site Q_i de la levure avec la structure reconstruite pour le *Plasmodium*, il semble que ce dernier ait un site Q_i un peu moins volumineux, surtout autour du substrat, avec plus d'espace occupé.

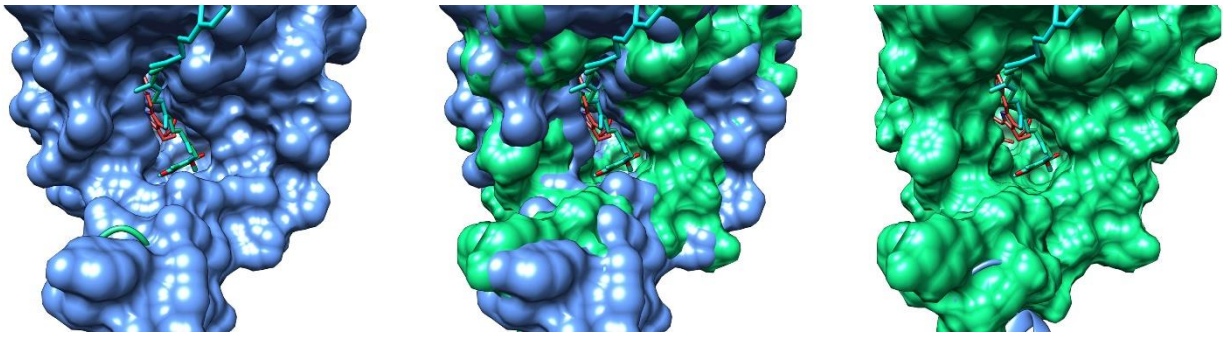

Figure 13. Comparaison de la structure du site Q_i entre *Plasmodium* (vert) et la levure (bleu).
De gauche à droite, le site Q_i de la levure, la superposition des deux structures et le site Q_i du *Plasmodium* modélisée. La quinone est colorée en cyan, l'hème en rouge. Structure PDB utilisée : 4PD4. La structure reconstruite du *Plasmodium* est modélisé à partir de l'alignement des séquences du cytochrome *b* de la levure et du parasite, en utilisant la structure cristallographique existante de la levure. Le module « MODELLER » du logiciel « Chimera » a été utilisé pour réaliser cette modélisation.

B.2. La cardiolipine et le passage du proton

La cardiolipine est un phospholipide anionique essentiel et spécifique de la mitochondrie. Cette molécule contient deux acides phosphatidiques qui se lient via le backbone de glycérol au centre pour former une structure dimérique. Elle contient 4 groupements alkyles et peut avoir 2 charges négatives. Selon l'espèce, la composition des groupements alkyles peut être différente. La cardiolipine représente environ 10-20% de la membrane interne. Elle a un rôle structural et fonctionnel. Le phospholipide s'associe aux complexes I , bc_1 et V (Schlame 2008) et avec le transporteur ADP/ATP (Claypool *et al.* 2008) et aide la formation des supercomplexes. Dans le complexe bc_1, le phospholipide serait aussi impliqué dans le passage du proton de la matrice vers le site Q_i (Haines and Dencher 2002). Avec ces fonctions structurelles et fonctionnelles, la cardiolipine peut augmenter considérablement l'activité globale de la chaîne OxPhos.

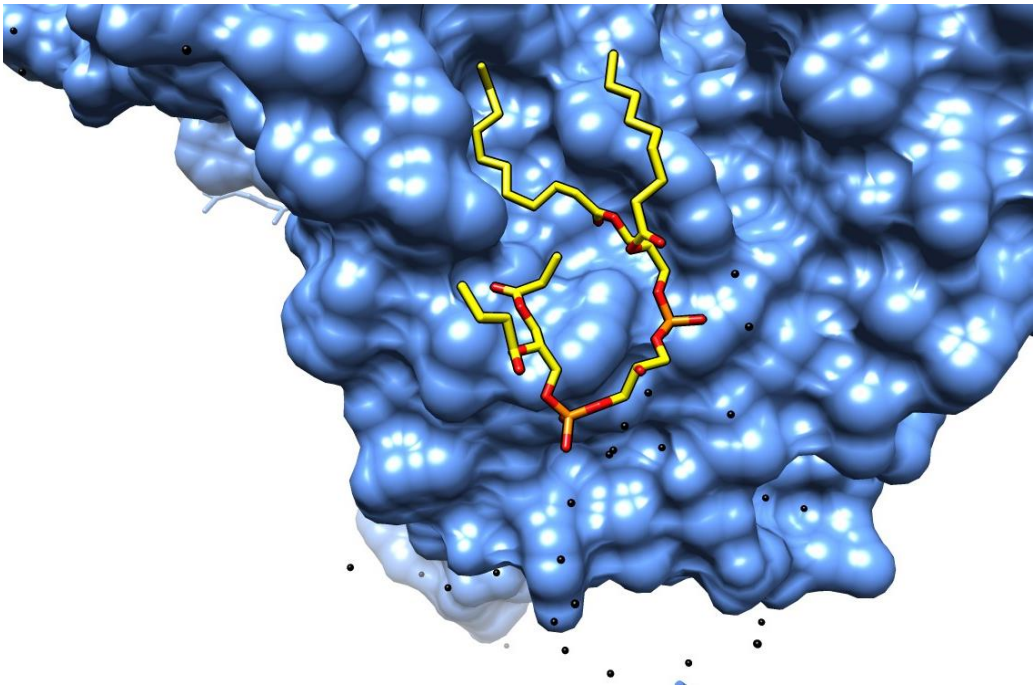

Figure 14. La cardiolipine liée à la surface du cytochrome *b*.
Le cytochrome *b* (surface) est coloré en bleu. La cardiolipine est en jaune. Les molécules d'eau sont en noir. La molécule de la cardiolipine s'associe bien avec les crêtes présentées à la surface du cytochrome *b*. Structure PDB utilisée : 1KB9.

Dans le complexe bc_1, la cardiolipine se lie fortement avec le cytochrome *b* à proximité du site Q_i (Figure 14). Dans les structures cristallographiques publiées, cette molécule est souvent observée liée de la même manière à l'extérieur du cytochrome *b*. Les lavages, lors la purification du complexe, ne permettent pas d'éliminer ce lipide. Pourtant, le exact rôle de la cardiolipine dans la formation du supercomplexe et le mécanisme du transfert de protons de la matrice vers le site Q_i ne sont pas encore clairs.

Deux voies de passage de protons vers le site Q_i ont été proposés : le passage E/R et le passage cardiolipine/K (Hunte, Palsdottir, and Trumpower 2003). Ces deux voies de passage se localisent dans deux extrémités du site Q_i. Ils pourraient fonctionner en même temps lors de la réduction des groupements carbonyles de l'ubiquinone sans que ce dernier se réoriente dans le site Q_i.

Dans le passage E/R, l'acide glutamique E52 de la sous-unité Qcr7p et l'arginine R218 du cytochrome *b* jouent un rôle clé. E52 se situe à l'interface hydrophile entre la sous-unité Qcr7p et le cytochrome *b*. Elle permet d'alimenter le passage avec des protons provenant de la matrice. R218, se situe à mi-chemin du passage et pourrait servir à faire avancer les protons via un réseau de liaisons hydrogènes dans le passage. Cette arginine est bien conservée et seulement remplaçable par une lysine (Hunte, Palsdottir, and Trumpower 2003).

Dans le passage cardiolipine/K, la lysine K228 est hautement conservée (Figure 15). Elle connecte les protons du site Q_i avec l'environnement aqueux à côté de la cardiolipine. Cette dernière se positionne juste devant l'entrée du passage qui donne directement vers un groupement carbonyle de l'ubiquinone. Le passage comporterait un réseau complexe de liaisons hydrogène formé par des molécules d'eau et des résidus hydrophiles. Les trois lysines conservées du cytochrome c_1 permettraient de stabiliser un des deux groupements phosphodiester de la cardiolipine par des liaisons hydrogène et peut-être ioniques. Dans le chapitre II-b, nous étudierons les mutations Y28S, N31D F225L, K228M et du cytochrome *b*. Ces

mutations se situent dans le passage de protons et modifieraient le réseau des liaisons hydrogène pour permettre un meilleur mouvement des protons vers le site Q_i.

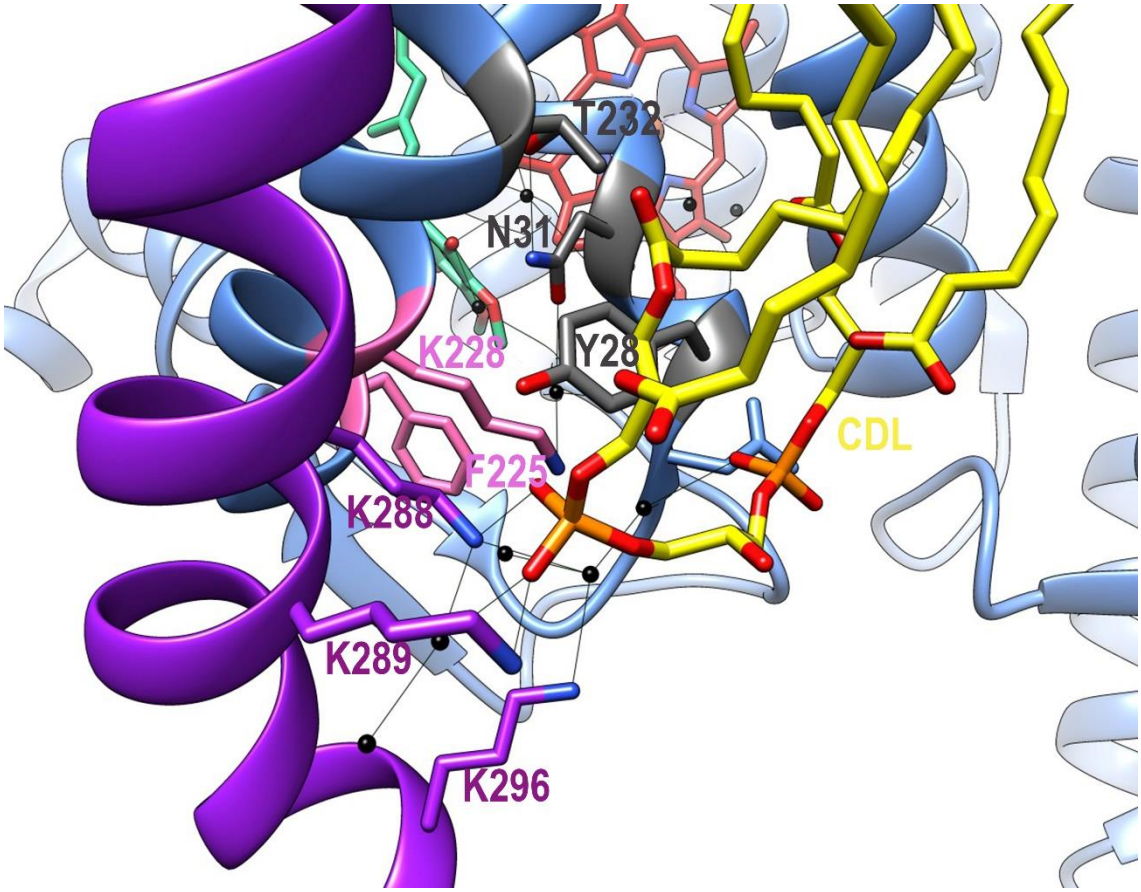

Figure 15. Voie de passage de protons cardiolipine/K228.
Le cytochrome *b* est coloré en bleu, le cytochrome c_1 en violet, la cardiolipine (CDL) en jaune, l'hème b_H en rouge, l'ubiquinone-6 en vert, K288, K289 et K296 du cytochrome c_1 en violet ; F225 et K228 du cytochrome *b* en rose et Y28, N31 et T232 du cytochrome *b* en gris. Un réseau très riche en liaison hydrogènes est montré par les lignes noires. Structures PDB utilisées : 1P84.

Résultats

Chapitre I. Études du site Q_o

Chapitre I. Études du site Q_o, la résistance et la sensibilité à l'atovaquone

Bien que le site Q_o soit relativement bien conservé entre les différentes espèces, les petites différences interspécifiques de séquences peuvent expliquer les sensibilités différentielles aux inhibiteurs. L'atovaquone, le seul antipaludique ciblant le complexe bc_1 sur marché jusqu'à présent, perd son efficacité à cause de mutations de résistance qui se développent au cours du traitement. Donc il est important de comprendre le mécanisme de l'apparition des mutations de résistance et aussi les conséquences, au niveau structural et fonctionnel, de ces mutations (chapitre I-A). Comme il y a des légères différences entre le site Q_o de levure et du *Plasmodium*, nous avons utilisé une souche de levure qui mime le site Q_o du parasite pour mieux caractériser l'effet de la mutation de résistance Y279S, qui est le plus souvent rapportée (chapitre I-B). Puisque beaucoup de polymorphismes humains dans le cytochrome *b* ont été observés, nous avons décidé d'étudier un panel de polymorphismes trouvés chez l'Homme introduits dans le cytochrome *b* de levure et d'analyser l'effet de ces mutations humaines sur la sensibilité aux inhibiteurs, spécialement à l'atovaquone (Chapitre I-C).

Chapitre I-A. Étude sur les souches Y279X

I. Introduction

Le résidu Y279 a un rôle clé pour la liaison à l'atovaquone. Son remplacement par d'autres acides aminés diminuerait fortement l'affinité de l'atovaquone pour le site Q_o. Trois mutations de résistance ont été reportées chez *P. falciparum* après échec thérapeutique : Y279S/C/N. Il semblait intéressant d'étudier pourquoi les autres changements d'acides aminés ne sont jamais observés.

Nous avons donc construit, chez la levure, une série de mutants portant des substitutions Y279X. Nous considérons que au cours du traitement avec de l'atovaquone, seulement les substitutions d'acide aminé résultant d'une seule substitution nucléotidique peuvent apparaître. Le codon pour Y279 chez la levure et pour Y268 chez le parasite est le même : UAU. Donc les possibilités de mutation sont : Y279C (UGU), D (GAU), F (UUU), H(CAU), N (AAU) et S (UCU), les autres donnent soit un codon d'arrêt soit une Y. Nous avons aussi décidé d'étudier Y279A et Y279W, même si ces substitutions nécessitent deux mutations nucléotidiques. Ces deux changements semblaient intéressants parce qu'une alanine a une courte chaîne latérale non polaire tandis qu'un tryptophane a une grande chaîne latérale aromatique et polaire. Ces propriétés peuvent nous aider à mieux comprendre l'interaction du résidu 279 avec les molécules qui se lient dans le site Q_o. Au total, nous avons étudié l'effet de 9 mutations.

J'ai mesuré la croissance respiratoire, l'activité du complexe bc_1, l'affinité vis-à-vis du substrat decylubiquinol, la production de superoxide (SO) et la sensibilité à différents inhibiteurs du site Q_o et Q_i. Avec des outils bio-informatiques, j'ai étudié la différence de structure dans le microenvironnement autour de la position 279 dans chaque mutant. En combinant les données biochimiques obtenues, j'ai pu répondre aux questions posées au début : Y279S/C/A induisent une résistance à l'atovaquone. À l'exception d'Y279F, toutes les mutations étudiées Y279S/C/A/H/W/N induisent une diminution d'activité du complexe bc_1 et ainsi qu'une chute de la vitesse de croissance respiratoire. En utilisant des alignements multiples pour toutes les séquences du cytochrome *b* du complexe bc_1 et du cytochrome b_6 du complexe b_6f, j'ai confirmé l'importance d'un acide aminé aromatique uni-cyclique à la position correspondante à 279 de la levure chez toutes les espèces séquencées. J'ai aussi observé que l'hydroxyle d'Y279 ne semble pas être nécessaire ou important pour la réactivité du site Q_o *in vivo* et *in vitro*. La liaison hydrogène que cet hydroxyle pourrait former avec H181 de l'ISP pourrait jouer un rôle dans la stabilité de la tête globulaire de l'ISP sur le site Q_o pour un transfert d'électron optimal. Mais dans notre étude, nous avons montré que sans avoir cette liaison hydrogène, le complexe bc_1 du mutant Y279F se comporte comme la souche sauvage.

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Yeast-based mutational analysis of the bc_1 complex Q_o site residue 279 to study the trade-off between atovaquone resistance and function

Zehua Song[1], Jérôme Clain[2, 3], Bogdan I. Iorga[4], Zhou Yi[1], Nicholas Fisher[5], and Brigitte Meunier[1]

[1] *Institute for Integrative Biology of the Cell (I2BC), CEA, CNRS, Université Paris-Sud, 91198 Gif-sur-Yvette, France*

[2] *UMR 216, Faculté de Pharmacie de Paris, Université Paris Descartes, 75006, Paris, France*

[3] *UMR 216, Institut de Recherche pour le Développement, 75006, Paris, France*

[4] *Institut de Chimie des Substances Naturelles, CNRS, UPR 2301, Labex LERMIT, 91198 Gif-sur-Yvette, France*

[5] *Plant Research Laboratory, Michigan State University, East Lansing, MI 48824, USA.*

Corresponding author:

Brigitte Meunier

Institute for Integrative Biology of the Cell (I2BC), CEA, CNRS, Université Paris-Sud, 1 avenue de la Terrasse, 91198 Gif-sur-Yvette, France

Tel 00 33 1 69823156

meunier@cgm.cnrs-gif.fr

Abstract

The bc_1 complex is central to mitochondrial bioenergetics and the target of the antimalarial drug atovaquone that binds in the Q_o site of the complex. Structural analysis has shown that the Q_o site residue Y279 (Y268 in *Plasmodium falciparum*) is key for atovaquone binding. Consequently, atovaquone resistance can be acquired by mutation of that residue. In addition to the probability of amino acid substitution, the level of atovaquone resistance and the loss of bc_1 complex activity that are associated with the novel amino acid would restrict the nature of resistance-driven mutations occurring on atovaquone exposure in native parasite populations. Using the yeast model, we characterized the effect of all the amino acid replacements resulting from a single nucleotide substitution at codon 279, Y279C, D, F, H, N and S. Two residue changes were added to the series, Y279A and W that required a double nucleotide substitution. We found

that mutations Y279A, C and S conferred high atovaquone resistance but decreased the catalytic activity. Y279F had wild-type enzymatic activity and sensitivity to atovaquone while the other substitutions caused a dramatic respiratory defect. The results obtained with the yeast model were examined at the light of the atomic structure and compared to the reported data on the evolution of acquired atovaquone resistance in *P. falciparum*.

Running title: Mutation of cytochrome *b* Y279

Keywords: respiratory complex III, atovaquone, yeast model, resistance, *Plasmodium*, malaria

Abbreviations used: Q_o, quinol oxidation site; Q_i, quinone reduction site; ISP, Iron-Sulfur Protein; SOD, superoxide dismutase; OD, optical density; DQ, decylubiquinol; TN, turnover number; IC_{50}, inhibitor concentration required to obtain 50% inhibition

Introduction

The mitochondrial respiratory chain complex III or bc_1 complex is central to mitochondrial bioenergetics and the target of antiprotozoals, such as atovaquone, and fungicidal drugs used to control human and plant pathogens. It is also currently the focus of intense research as an antimalarial target for further drug development (1-6). The bc_1 complex is a multimeric enzyme. Three subunits form the electron-transferring catalytic core and contain the redox-active groups, namely cytochrome *b*, cytochrome c_1 and the 'Rieske' iron sulphur protein (ISP). Cytochrome *b* is mitochondrially encoded in all eukaryotes and contains the substrate ubiquinol/ubiquinone binding sites (Q_o and Q_i sites), which form the sites of competitive inhibition for antimicrobial agents. In the context of malaria, atovaquone (used in combination with proguanil, and marketed as Malarone®) is a popular prophylactic drug and also shows high efficiency in the treatment of uncomplicated *Plasmodium falciparum* malaria.

Interestingly, atovaquone resistance frequently evolves through *de novo* cytochrome *b* mutations during antimalarial therapy (7-10). The resistance is caused by the mutation of residue Y279 (Y268 *P. falciparum* numbering) in the atovaquone target site, the bc_1 complex Q_o site. Y279 is a highly conserved residue. It is crucial for stabilizing the bound atovaquone (Birth, Kao, and Hunte 2014) and is postulated to play a key role in the binding and correct positioning of the ubiquinol in the Q_o site facilitating a fast electron transfer to the [2Fe-2S] cluster of the ISP (12, 13).

In *P. falciparum*, two substitutions of Y279 are commonly associated with atovaquone resistance *in vivo*, namely Y279S and Y279C (7, 9) whereas a third mutation, Y279N, is much less frequent (Sutherland *et al.* 2008). These three amino acid replacements correspond to single nucleotide substitutions at codon 279, suggesting that the evolutionary space is restricted to easily accessible amino acid replacements. Therefore we hypothesized that the *in vivo* evolution of *P. falciparum* resistance to atovaquone results from a balance between various properties of residue 279: the probability of amino acid substitution, the level of atovaquone resistance and the possible loss of bc_1 complex activity that are associated with the novel amino acid.

As *Plasmodium* parasites are not amenable to mitochondrial transformation, the effect of the different mutations (other than those observed in the wild) of Y279 on bc_1 complex activity and sensitivity to drugs

cannot be assessed directly and an experimental model has to be used. Yeast (*Saccharomyces cerevisiae*) is amenable to mitochondrial transformation. Its cytochrome *b* shares a high level of sequence similarity with the parasite cytochrome *b*. Therefore yeast provides a useful surrogate model to study the functional effect of mitochondrially-encoded cytochrome *b* mutations. In addition, yeast can be grown in either respiratory or fermentative conditions, which facilitates the production of mutants with highly deleterious respiratory effects and their subsequent analysis. Following that strategy, Y279S and C yeast mutants have been previously produced and shown to combine high resistance to atovaquone and decreased activity (15, 16).

Here, in order to fully explore the mutational landscape of Y279 associated with atovaquone resistance, we studied, in the yeast model, the effect of all the amino acid replacements resulting from a single nucleotide substitution at codon 279, namely Y279C, D, F, H, N and S. To further explore the biochemical requirements at residue 279, we also analysed two additional mutations, Y279A and Y279W, which introduce a small hydrophobic and a bulky aromatic residue, respectively, although the amino acids replacement required a double nucleotide change of codon Y279.

We measured the effect of these mutations on the respiratory growth competence and on the bc_1 complex activity and sensitivity to inhibitors. Structural modelling was then used to examine the impact of the amino acid changes on the structure and function of the Q_o site.

Materials and Methods

Materials and growth media

Equine cytochrome *c*, decylubiquinone, azoxystrobin, antimycin, atovaquone, superoxide dismutase, catalase and NADH were obtained from Sigma Aldrich. The following media were used for the growth of yeast: YPD (1% yeast extract, 2% peptone and 3% glucose), YPGal (1% yeast extract, 2% peptone and 2% galactose), YPeth (1% yeast extract, 2% peptone and 2% ethanol) and YPG (1% yeast extract, 2% peptone and 3% glycerol).

Yeast mitochondrial mutants. The mutants were generated by side-directed mutagenesis and mitochondrial transformation as described in (Hill *et al.* 2003). They have identical nuclear and mitochondrial genomes with the exception of the mutations introduced in the cytochrome *b* gene.

Measurement of NADH- and decylubiquinol-cytochrome *c* reductase activity. Yeast mitochondria were prepared as in (Lemaire and Dujardin 2008). Briefly, yeast grown in YPGal medium were harvested at mid-log phase. Protoplasts were obtained by enzymatic digestion of the cell wall using zymolyase in an osmotic protection buffer. Mitochondria were then prepared by differential centrifugation following osmotic shock of the protoplasts. Mitochondrial samples were aliquoted and stored at -80 °C. Concentration of bc_1 complex in the mitochondrial samples was determined from dithionite-reduced optical spectra, using ε=28.5 mM^{-1} cm^{-1} at 562 nm *minus* 575 nm. NADH-, and decylubiquinol-cytochrome *c* reductase activities were determined at room temperature by measuring the reduction of cytochrome *c* (final concentration of 20 µM) at 550 nm *versus* 540 nm over 1-min time-course in 10 mM potassium phosphate pH 7 and 1 mM KCN. Lauryl-maltoside (0.01% (w/v)) was added to the

reaction buffer for the decylubiquinol-cytochrome *c* reduction assays. Mitochondria were added to obtain a final concentration of 5-30 nM bc_1 complex. Activity was initiated by the addition of decylubiquinol (final concentration: 5-20 μM), a synthetic analog of ubiquinol or by the addition of NADH (final concentration: 100 μM). Initial rates were measured. The measurements were repeated three to five times and averaged. Turnover numbers (TN) were determined as the cytochrome *c* reduction rate per bc_1 complex at 20 μM decylubiquinol or 100 μM NADH. K_M values were estimated from the plots of cytochrome *c* reduction rates *vs* decylubiquinol concentrations. The mid-point inhibition concentrations (IC_{50}) were determined by inhibitor titration.

Ligand docking and molecular modelling. Three-dimensional structure of decylubiquinol was generated using CORINA, Molecular Networks GmbH, Erlangen, Germany (http://www.molecular-networks.com). The structure of the yeast bc_1 complex was downloaded from the Protein Data Bank (Berman *et al.* 2000) and used as receptor in the docking process. Water molecules, ligands, lipids and haems were removed, as well as all other chains except those corresponding to cytochrome *b* and ISP. Hydrogen atoms were added using HERMES, the graphical interface of GOLD (Verdonk *et al.* 2003). The docking of decylubiquinol was performed with GOLD using a binding site (Q_o site) defined as a 15Å radius sphere centered on the δ1-carbon atom of cytochrome *b* residue I147. GoldScore was used as a scoring function and all other parameters had default values. *In silico* mutations Y279X were introduced using CHIMERA (Pettersen *et al.* 2004), which was also used for generating the molecular modeling images.

Results

Effect of substitution of Y279 in yeast bc_1 complex on the enzymatic activity and atovaquone-sensitivity

Residue Y279 has a key role in binding atovaquone in the bc_1 complex Q_o site (Birth, Kao, and Hunte 2014). Therefore it is reasonable to expect that substitution of this residue may confer resistance to the drug. Here we studied the functional impact of all amino acid replacement resulting from a single nucleotide substitution of codon Y279 (UAU) in yeast cytochrome *b*, namely Y279C (UGU), Y279D (GAU), F (UUU), H (CAU), N (AAU), and S (UCU), because these are the most likely to occur *in vivo* during malaria therapy. Two amino acid replacements resulting from a double nucleotide substitution were also analysed, Y279A and Y279W. They introduce a small hydrophobic and a bulky aromatic residue, respectively.

The mutants were generated as described in (Hill *et al.* 2003).The respiratory growth competence (Fig 1), the bc_1 complex activity measured as decylubiquinol- and NADH-cytochrome *c* reductase activities, and the sensitivity to Q_o and Q_i site inhibitors were then monitored (Table 1).

Whereas all the mutants grew as well as the wild-type (wt) in fermentative medium, in which the cell energy does not depend on a functional mitochondrial respiratory chain, large differences in respiratory growth rate were found among the mutant strains.

Mutations Y279D, H, N, W caused a complete respiratory growth defect. Y279D was not studied further. Y279H, N and W had lost around 95% of the control bc_1 complex activity and the resistance to atovaquone could not be tested. As the introduction of charged or bulky residues at position 279 might

destabilise the interaction with the ISP (which has been shown to be labile in some mutant strains (Bratton *et al.* 2003)), we monitored the level of that subunit in the different mutants. It was previously shown that the replacement of the tyrosine by serine did not result in the loss of ISP (Kessl *et al.* 2005). By Western blot analysis, we confirmed that data and found that the level of ISP was not decreased in any of the mutants studied here (not shown). Thus the mutations resulted in an assembled but inactive enzyme.

Y279C and S have been reported in *Plasmodium falciparum* and shown to cause atovaquone resistance (see for instance (7, 9)). The same substitutions introduced in the yeast enzyme also conferred atovaquone resistance (around 45-fold resistance compared to wt). The mutated complexes had a four-fold lower decylubiquinol- and NADH-cytochrome *c* reductase activities and the respiratory growth competence of the mutants was decreased. Y279A also combined atovaquone resistance with a decreased activity. This mutant, however, had a severe defect in respiratory growth due to the loss of more than 80% of the bc_1 complex activity. We have previously observed that there is a sharp fall in respiratory growth competence between 25 and 15% of bc_1 complex activity (unpublished data).

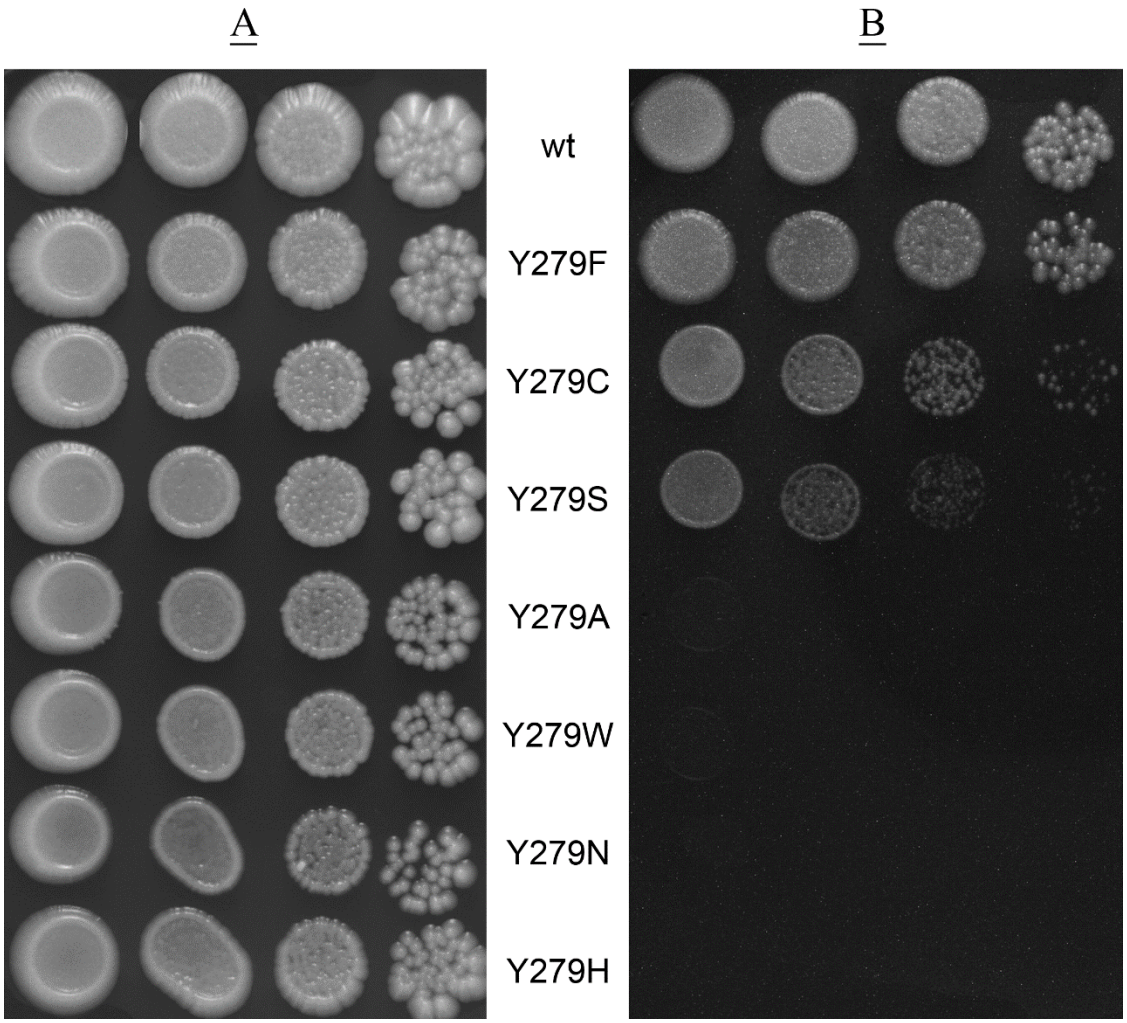

Figure. 1. Respiratory growth competence of Y279 mutants. Serial dilutions in water of cells pre-grown on glucose plates were spotted on plates containing either glucose (A: YPD, fermentative medium) or glycerol (B: YPG, respiratory medium) and incubated for three days at 28 °C.

By contrast, mutation Y279F had minimal effect on growth, bc_1 complex activity or atovaquone sensitivity, except for a slightly higher K_M for decylubiquinol. The TN/K_M ratio (effectively a second-order rate constant for the interaction between substrate and enzyme) was slightly lower in the mutant, 13.6 and 17 for Y279F and wt, respectively, which indicates a mild decrease of the catalytic efficiency of the mutant enzyme.

As a control, we monitored the sensitivity of the wt and mutant enzymes to antimycin and azoxystrobin, two well-known bc_1 complex inhibitors that bind at different target sites. Y279F, C, S and A, showed the same sensitivity than wt to the Q_i site inhibitor antimycin. They also presented a very similar sensitivity to azoxystrobin that binds in the Q_o site but in position different to atovaquone, (*i.e.* more proximally to haem b_L.(Esser *et al.* 2004)) with the exception of Y279S that showed a small 2.7-fold resistance increase.

Residue *cytb*^{279}		Growth [a]	Decylubiquinol cyt *c* reduction [b] (mean ± SD)					NADH cyt *c* Reduction [b]
Codon [d°]	Amino acid [e]		Turn-over	K_M	IC_{50}/bc_1 [c]			Activity
		%wt	% wt	µM	atovaquone	azoxystrobin	antimycin	% wt
UAU	Y279	100 ± 5	100 ± 1	3.1 ± 0.32	4 ± 0.6	12 ± 1.2	0.3 ± 0.01	100 ± 5
U**U**U	Y279F	94 ± 4	98 ± 2	4.9 ± 0.40	2 ± 0.2	11 ± 0.7	0.3 ± 0.02	95 ± 2
U**G**U	**Y279C**	27 ± 2	24 ± 1	3.0 ± 0.15	180 ± 21	15 ± 0.7	0.3 ± 0.02	28 ± 1
U**C**U	**Y279S**	17 ± 3	24 ± 1	3.6 ± 0.15	193 ± 12	33 ± 2.1	0.2 ± 0.01	33 ± 1
GCU	Y279A	1 ± 0.5	18 ± 1	3.2 ± 0,12	154 ± 11	18 ± 2.2	0.4 ± 0.02	14 ± 0.2
GAU	Y279D	0	nd	nd	nd	nd	nd	nd
CAU	Y279H	0	6	nd	nd	nd	nd	nd
AAU	**Y279N**	0	6 ± 0.5	nd	nd	nd	nd	5 ± 1
U**GG**	Y279W	0	6 ± 0.4	nd	nd	nd	nd	6 ± 0.4

Table 1. Effects of Y279 mutations on respiratory growth and bc1 complex activity and sensitivity to inhibitors

[a] Growth competence was monitored in YPEth medium. Culture started at an OD (measured at 600 nm) of 0.5. They were incubated with vigorous agitation for three days at 28 °C. The OD were then recorded. The values are presented as % of the wt OD.

[b] Cytochrome *c* reduction activity measured as described in Materials and Methods and reported on the bc_1 complex concentration. The values are presented as % of the wt activity. wt decylubiquinol cytochrome *c* reduction rate was 53 ± 1.4 s^{-1} (mean value ± standard deviation); wt NADH cytochrome *c* reduction rate was 106 ± 5.1 s^{-1} (mean value ± standard deviation)

nd: not determined. cyt *c*: cytochrome *c*

[c] IC_{50}/ bc_1, inhibitor concentration required to obtain 50% inhibition reported on the bc_1 complex concentration

All the measurements have been made three to five times (except for Y279H) and averaged.

[d] Mutated bases are shown in bold and underlined letters.

[e] Amino acid at position 279 of cytochrome *b* (yeast numbering, which corresponds to the position 268 in *P. falciparum*) found to be associated with clinical atovaquone resistance in *P. falciparum* are shown in bold.

Finally, we tested the production of superoxide by the wt and mutant bc_1 complexes. Mutation of the Q_o site may cause catalytic cycle dysfunction that results in side-reactions and superoxide (SO) production. We measured SO production by monitoring the superoxide dismutase (SOD)-sensitive rate of cytochrome *c* reduction. The difference between the reduction rate in the absence and the presence of added SOD gives the contribution of the cytochrome *c* reduction by SO to the overall cytochrome *c* reductase activity. We compared the effect of the mutations Y279F, C, S, A on SO production. The data are shown in Fig. 2.

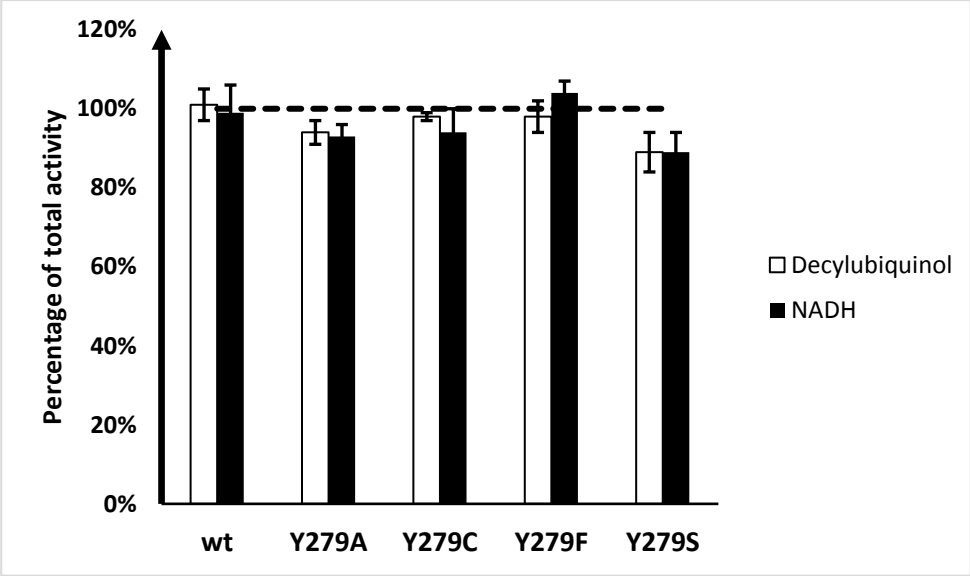

Figure. 2. Superoxide production in wild-type and mutants. Cytochrome *c* reduction rates were recorded in absence and in presence of SOD and catalase, both at 225 units/mL, using 20 µM of decylubiquinol or 100 µM of NADH as substrates. The assays were performed as described in Materials and Methods, with slight modifications made to optimize the SOD and catalase activities: decylubiquinol-cytochrome *c* reduction were assayed in potassium phosphate buffer 50 mM pH 7 with 10 µM KCN; NADH- cytochrome *c* reduction was monitored in potassium phosphate buffer 10 mM pH 7.4 with 5 µM KCN. Each measurement was repeated at least three times and averaged. The decylubiquinol- (white) and NADH – (black) cytochrome *c* reduction rates in presence of SOD and catalase are presented as percentage of the rates in absence of SOD-catalase.

The wt and mutant Y279F bc_1 complexes did not produce SO. No or little SO production was detected for Y279C. Around 11% and 7% of the cytochrome *c* reduction activity of the enzyme could be attributed to SO in Y279S and Y279A, respectively. It was previously shown that Y279A, C and S mutant enzymes exhibited a low SO production, very similar to that observed in this study (Wenz *et al.* 2007). Similar observations were reported using *Rhodobacter capsulatus* Y279 mutants (Lee *et al.* 2011).

The increase in SO production can be understood in terms of higher semiquinone occupancy at the Q_o site, increasing the equilibrium concentration of semiquinone for reaction with oxygen or an increased rate constant for the reaction with oxygen, perhaps by steric means (*i.e.* an increased probability of

collision with oxygen). SO production observed in the Y279A and Y279S mutants in this study may reflect a prolonged occupancy of the semiquinone in the Q_o site and a slower semiquinone oxidation by the normal reaction partner, ferrihaem b_L.

In summary, of the eight substitutions of Y279 studied here, Y279D, H, N and W had a dramatic effect on the respiratory function. Y279A, C and S significantly decreased the bc_1 complex activity while Y279F resulted in a fully functional enzyme. The differential effect of the substitutions on the enzymatic activity could be explained by examining the Q_o site structure.

Residue 279 in the Q_o site structure

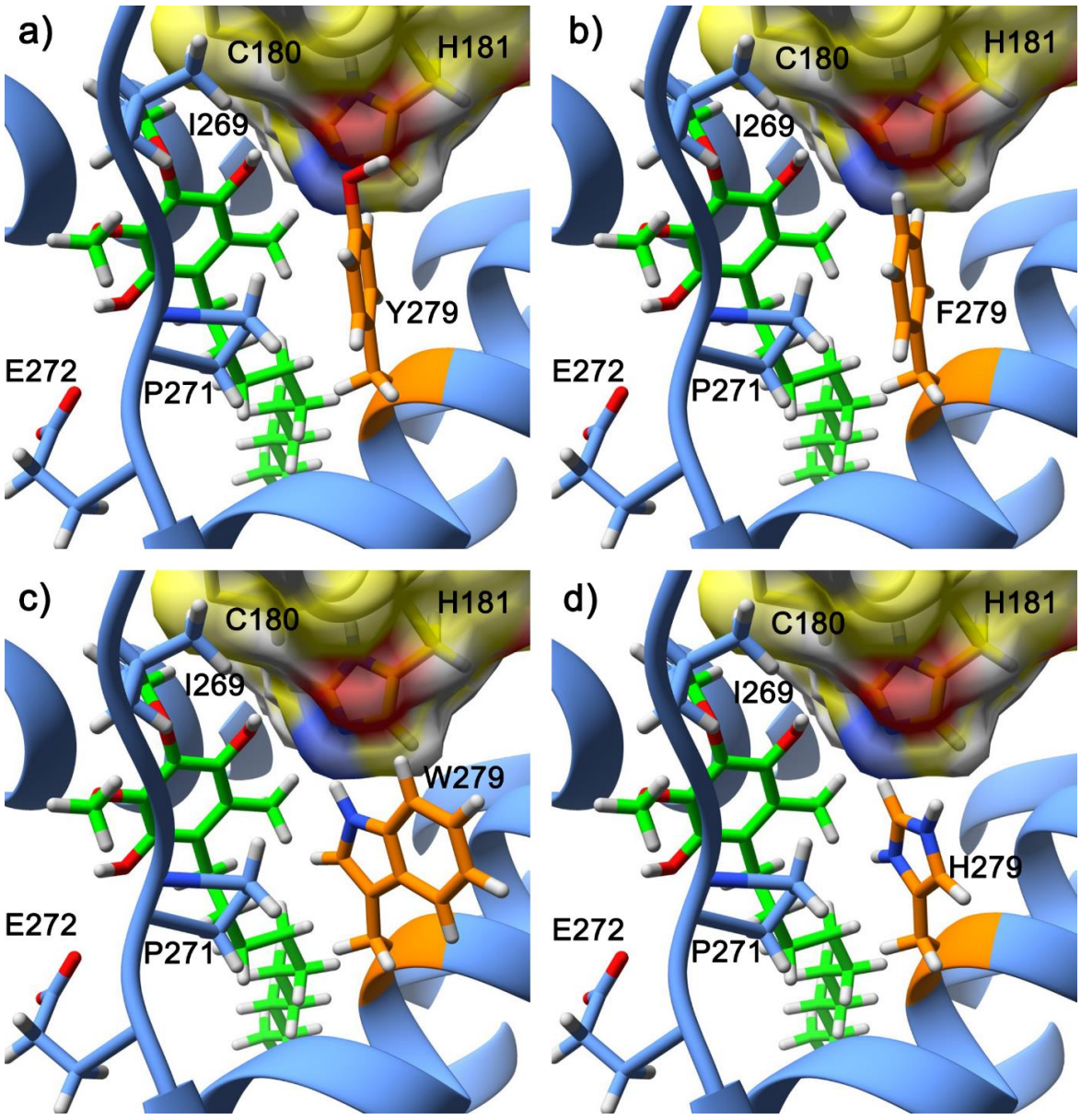

Figure. 3. Location of residue 279 in the *bc*$_1$ complex Q_o site. (a) Wild-type aminoacid Y279 in contact with P271 of the PEWY motif. (b-d) Substitution of Y279 by the aromatic residues F279 (b), W279 (c) and H279 (d). Cytochrome *b* is presented in blue. ISP is in yellow with its surface colored by heteroatoms. Decylubiquinol is in green. Residue 279 is in orange. Oxygen atoms are colored in red, nitrogens in dark blue and hydrogens in light grey. The model has been constructed as described in Materials and Methods.

An atomic structure for ubiquinol bound within the Q_o site is not available. Nevertheless, the structure of the stigmatellin-inhibited enzyme is considered to provide a good model for the quinol binding at the Q_o site prior to the formation of the semiquinone anion and electron transfer to ferrihaem b_L (PDB code 3CX5 (Solmaz and Hunte 2008)). Starting from that atomic structure, we also generated *in silico* models of the quinol-bound Q_o site (Fig.3). These were used to examine the role of Y279 in quinol binding and to evaluate the impact of the substitutions of that residue.

The tyrosyl sidechain of Y279 is likely to contribute to quinol binding in different ways. The residue makes a significant (65 $Å^2$) hydrophobic interaction with the chromone headgroup of stigmatellin, and is likely to perform a similar function with the native quinol substrate.

The hydroxyl of Y279 could also form a hydrogen bond with the oxygen atom of ISP residue C180, which might act to stabilise the mobile domain of the ISP at the surface of the Q_o site, facilitating quinol oxidation. However, that hydrogen bond seems to have only a modest role as the replacement of tyrosine by phenylalanine has little effect on the bc_1 complex function as shown in Table 1, which is in agreement with previous studies using bacterial enzymes (25, 27).

Y279 is also likely to contribute to the correct conformation of the *ef* loop comprising the highly conserved motif PEWY $_{271\text{-}274}$ and the *ef* helix $_{275\text{-}284}$, which would be required to maintain the correct structure of the catalytic cavity and to ensure an optimal binding of the substrate quinol (see (E. A. Berry and Huang 2011) for a review). As presented in the model (Fig. 3a), Y279 is in the immediate vicinity of P271, part of the PEWY motif and conserved in almost all organisms (Kao and Hunte 2014). The proline ring and the tyrosine ring are in van der Waals contact. Their interaction might be important to maintain the optimal distance between I269 and ISP C180 (and thus the correct docking of the ISP), and between residue E272, key player in quinol oxidation (30), and the quinol.

Substitution of Y279 by non-aromatic residues would result in the loss of these stabilising interactions and compromise the quinol binding and oxidation. This explains the loss of bc_1 complex activity resulting from the mutations Y279A, C, D, N, and S. The severity of the activity loss was found to increase in the following order: C>S>A>N/D. In bacteria, analysis of mutant bc_1 complexes showed that replacement of Y279 by valine or leucine had a moderate effect on the enzyme activity; alanine and cysteine increased the severity; serine and glycine caused further activity loss. The most severely impaired mutant seemed to be Y279Q (25, 27). It thus appears that, in yeast as in bacterial complex, the severity of the effect is determined by both the size and the polarity of the side-chain.

The effect of the substitution of Y279 by an aromatic residues was then examined. Based on the structural model (Fig.3), it appeared that the aromatic residue phenylalanine (Fig.3b) could fulfill the role of Y279, which explains that the substitution Y279F had no or very mild effect. By contrast, the introduction of the bulkier aromatic residue tryptophan (Fig.3c) is likely to result in steric hindrance impairing the structure of the Q_o site and thus its function. Substitution of the tyrosine by the charged residue histidine (Fig.3d) would alter the environment of the quinol binding site. It is not tolerated in yeast Q_o site as the mutation causes a dramatic loss of bc_1 complex activity.

The importance of an aromatic residue, tyrosine or phenylalanine, in position 279 is supported by the conservation of the residue amongst species. Using a large collection of available cytochrome *b* sequences (over 9500), we monitored the nature of the residue at the fifth position after the conserved PEWY motif, which corresponds to residue Y279 in yeast cytochrome *b* (Supplemental data Table S1).

A tyrosine is conserved in the cytochrome *b* of almost all bacteria, fungi and protozoa and in all of animals. In all the *Plasmodium* sequences available in GenBank database, the conservation of Y279 is respected besides sequences reported from clinical atovaquone-resistant cases, in which a serine was observed at this position. In Archaea, tyrosine and phenylalanine are found in, respectively, 61% and 39% of the analyzed sequences. A tyrosine is also observed in 69% of plant cytochrome *b* while, in the remaining 31% of the available sequences, a histidine replaced the tyrosine at position 279. This is intriguing as in yeast, the substitution of tyrosine by histidine results in an inactive enzyme. It may be postulated that plant Q_o site has evolved for a better compatibility with a histidine and that the presence of this charged residue might be compensated by other variations. Comparison of sequences of the amino-acids in the vicinity of residue 279 did not reveal major differences, suggesting more distant or subtle effects. In all b_6f complex subunit IV, a phenylalanine is found instead of a tyrosine. Subunit IV with cytochrome b_6 shows many structural and functional similarities with bc_1 complex cytochrome *b*. Comparison of the structure of the bc_1 and b_6f complexes Q_o sites shows that the Y279 and the equivalent phenylalanine occupy very similar position and orientation (not shown).

Thus, the data indicate that –with the exception of some plants- the correct function of the Q_o site requires the aromatic residues tyrosine or phenylalanine.

Discussion

The anti-malarial drug atovaquone binds in the b_L-distal region of the Q_o site, similarly to stigmatellin, and forms a strong hydrogen bond (2.8 Å) to the protonated imidazole ring of the ISP [2Fe-2S] cluster H181 in the recently published yeast atomic structure (PDB code 4PD4 (Birth, Kao, and Hunte 2014)). The tyrosyl sidechain of residue Y279 (Y268 in *P. falciparum*) makes a significant, stabilising aromatic contact with the hydroxynaphthoquinone moiety of Q_o-bound atovaquone. That aromatic interaction can also be formed by a phenylalanine. Other substitutions resulting in the loss of that key stabilising interaction would thus cause atovaquone resistance which is associated with atovaquone-proguanil treatment failure in malaria.

Using the yeast model, we studied eight substitutions of Y279. Six of these amino-acid replacements, Y279C, D, F, H, N and S, resulted from a single nucleotide substitution of codon 279 while two, Y279A and W, required a double nucleotide substitution. The consequences of the mutations on the respiratory growth competence and on the bc_1 complex activity and sensitivity to atovaquone were investigated. Then the structural basis of the effects of the mutations was examined.

The substitution Y279F had no or little effect on bc_1 complex activity and sensitivity to atovaquone. This was expected from the examination of the Q_o structures that indicates that tyrosine and phenylalanine could be swapped without deleterious effect and without loss of the stabilising interaction with atovaquone. Four mutations (Y279D, H, N and W) lead to a dramatic loss of respiratory function. The atovaquone sensitivity could not be tested. Three substitutions (Y279A, C and S) resulted in atovaquone resistance combined with decreased activity.

Acquisition of atovaquone resistance in malaria parasites needs three requirements to be fulfilled: high probability of the amino acid replacement, high level of atovaquone resistance, and a limited loss of fitness associated with the resistance mutation. Based on the yeast analysis, three mutations of Y279 fulfil the atovaquone resistance and fitness criteria, Y279C, Y279S, and Y279A. However, whereas Y279C and Y279S amino acid replacements result from a single nucleotide substitution, Y279A requires a double

nucleotide substitution which makes it unlikely to occur on the short time scale of a malaria treatment. This is fully consistent with Y279A not being found in any of the atovaquone-resistant parasites reported to date, and with Y279C and Y279S being the two major mutations found in atovaquone-resistant parasites from atovaquone-proguanil treatment failures (7, 9).

A third mutation, Y279N, has been reported in association with clinical atovaquone resistance (7, 9, 14). Based on the data obtained with the yeast model, that mutation would not be expected to be tolerated as it causes a severe loss of function. However, reported studies indicate that, in the parasite as in yeast, Y279N has a more deleterious impact that Y279C and Y279S. First, analysis of rodent malaria parasites showed reduced *in vivo* growth rate and bc_1 complex activity for Y279N mutant strains when compared to Y279C (31, 32). Then, a heavier fitness penalty is suggested by the rate at which the atovaquone-resistance mutations emerged and were selected during malaria therapy with Y279S, Y279C, and Y279N representing roughly 54%, 42%, and 4% of mutant occurrence, respectively (n=11, 14, and 1 for Y279C, Y279S and Y279N, respectively; see (Cottrell *et al.* 2014) Supplementary Table 2). Minor structural differences in the Q_o site structure between the parasite and yeast enzymes might result in a more stringent effect of Y279N in the yeast enzyme.

In the parasite - as in yeast - the acquisition of atovaquone resistance is associated with loss of bc_1 complex activity and decreased fitness (Nicholas Fisher *et al.* 2012). The resistance mutations would be expected to be counter-selected in the absence of drug pressure and thus not detected in the parasite cytochrome *b* gene of field isolates. Atovaquone-proguanil being poorly used in endemic areas because of its high cost, it is therefore intriguing that the mutations Y279C, S and N have been reported in field isolates from Kenya at rather high frequency (Ingasia *et al.* 2015). However, as the level of drug use was not documented, interpretations of these data should be taken with caution. It might be hypothesized that other polymorphisms in the Q_o domain, in cytochrome *b* or ISP, partly compensate the loss of the tyrosine. The genetic compensation would result in a more active enzyme and a reduced fitness cost. In yeast, we found that changes in the ISP hinge region could partially restore the respiratory function severely altered by the Q_o site mutations G291D, S152P and A144F (22, 35).

The bc_1 complex is an attractive target for antimicrobial drugs. Atovaquone is currently the sole drug in clinical use targeting that enzyme. Here we analysed in the yeast model the trade-off between atovaquone resistance and bc_1 complex activity that associates with mutations or Y279. We showed that cysteine and serine are optimal atovaquone-resistant amino acid at position. These results parallel the *in vivo* evolution of atovaquone resistance during malaria therapy and support the yeast model as a useful surrogate to study anti-malarial drug resistance.

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Supplementary data

	Number of sequences	Variance
Cytochrome *b*		
Archaea	147	Y (61%), F (39%)
Bacteria	1809	Y (99.2%)
Fungi and protozoa	306	Y (95.44%)
Plasmodium	1997	Y (99.8%)
Animals	4744	Y (99.494%)
Plants	123	Y (68.55%), H (31.45%)
Cytochrome b_6f		
Plants	395	F (100%)

Table S1. Conservation of residue 279. The variance of the fifth residue after PEWY motif (equivalent of residue Y279 in yeast cytochrome *b*) was checked in bc_1 complex cytochrome *b* sequences and in b_6f complex subunit IV sequences. Sequences were retrieved from "RefSeq" database (on the 19th January 2015). The *Plasmodium* sequences were retrieved from "GenBank" database (on the 19th January 2015). Alignments of the sequences were based on the PEWY motif using Clustal Omega. Default parameter was kept and results were analyzed using Jalview and a python script.

III. Discussion

Les substitutions possibles résultant d'un seul changement nucléotidique du codon pour Y279 sont : phénylalanine, cystéine, sérine, acide aspartique, histidine et asparagine (F, C, S, D, H et N). F n'a pratiquement pas d'effet par rapport au sauvage (Y) à la position 279 au niveau de l'activité du complexe bc_1 et de la croissance respiratoire, à part une plus grande sensibilité à l'atovaquone. Cette mutation ne serait donc pas sélectionnés pendant le traitement avec de l'atovaquone.

H et D en position 279 annulent l'activité respiratoire, chez la levure, ce qui peut expliquer pourquoi ces changements n'ont jamais été observés chez le parasite.
C et S ont souvent été découverts dans les échecs cliniques au cours du traitement, et sont supposées d'être responsables de la résistance au médicament. Notre étude confirme que ces deux autres substitutions peuvent aussi induire une résistance à l'atovaquone chez la levure et sont toujours compatibles avec un complexe fonctionnel.

N a aussi été reporté chez les parasites trouvés dans les échecs thérapeutiques mais bien moins fréquemment que C et S. Ce changement, chez la levure, cause une sévère diminution de l'activité du complexe. Cette mutation aurait un effet moins délétère chez le parasite. Peut-être les petites différences de structures des sites Q_o de levure et de *Plasmodium* expliquent cette différence d'effet. Nous pourrions aussi poser l'hypothèse que, dans les rares cas où ce changement est apparu, les souches avaient d'autres modifications du site Q_o qui permettaient d'accommoder la mutation Y279N sans une perte trop sévère de l'activité du complexe. Il serait intéressant d'analyser la région charnière de l'ISP car des mutations compensatoires dans cette région peuvent restaurer une meilleure activité du complexe quand sa fonction est altérée par des mutations du cytochrome *b*, chez la levure (Brasseur *et al.* 2004). Des parasites portant des polymorphismes dans la région charnière pourraient développer une résistance à l'atovaquone plus facilement.
Nous avons observé que seules Y et F peuvent garantir une bonne activité enzymatique. Il semble donc qu'un acide aminé monocyclique non chargé soit requis à la position 279 pour que l'enzyme fonctionne bien. Un acide aminé trop petit (S, C, A, N), un acide aminé trop grand (W) ou un acide aminé chargé (H) ne permettent pas son bon fonctionnement.

L'atovaquone et l'ubiquinol partagent probablement la même position de liaison dans le site Q_o. Y279 joue un rôle très important dans la liaison de ces deux molécules. Donc une mutation d'Y279 pourrait soit ne pas avoir d'effet majeur sur l'affinité à l'enzyme des deux molécules, soit déstabiliser la liaison des deux molécules. Les résultats récents de (Goodman *et al.* 2016), obtenus chez le parasite, ont abouti à la même hypothèse. Ils ont démontré que lorsque certaines mutations (y compris Y268S/N, numérotation du parasite, équivalentes d'Y279S/N chez la levure) confèrent une résistance à l'atovaquone chez le parasite, cette résistance rend la chaîne respiratoire peu active. L'augmentation de la résistance permet aux parasites de survivre lors du développement dans des cellules de l'hôte où la chaîne respiratoire ne fonctionne pas à pleine vitesse. Par contre, lors du développement chez les moustiques où la chaîne respiratoire fonctionne à fond, la diminution de la respiration inhibe plus fortement la croissance parasitaire. Et cette croissance inhibée empêche la transmission des parasites et ainsi la dispersion de la mutation. Cela a mis en valeur le cytochrome *b* comme cible thérapeutique pour lutter contre le paludisme et d'autres apicomplexes pathogènes.

La liaison hydrogène entre le groupement hydroxyle d'Y279 du cytochrome *b* et H181 de l'ISP reporté avant ne semble pas jouer un rôle important dans l'activité enzymatique. Il est intéressant de noter que dans le cytochrome *b* de certaines archées et de plantes, à la position correspondant à Y279 chez la levure, F ou H est présent. Le substrat utilisé et le potentiel membranaire varient en fonction des espèces (Kao and Hunte 2014) chez des archées et des plantes. Dans ces espèces, une histidine ou une phénylalanine serait mieux adaptée au fonctionnement du complexe et cela pourrait donc expliquer pourquoi Y279 n'est pas totalement conservée. Chez les plantes, dans le complexe b_6f, structuralement similaire au complexe bc_1, une F est 100% conservée. Le complexe b_6f est structuralement similaire au complexe bc_1, surtout au site Q_o (Figure 16). Les structures cristallographiques de ce complexe ont été publiées. La structure correspondant au cytochrome *b* est composée de deux sous-unités : le cytochrome b_6 et la sous-unité IV. Le site Q_o est localisé dans le cytochrome b_6 et le site Q_i dans la sous-unité IV.

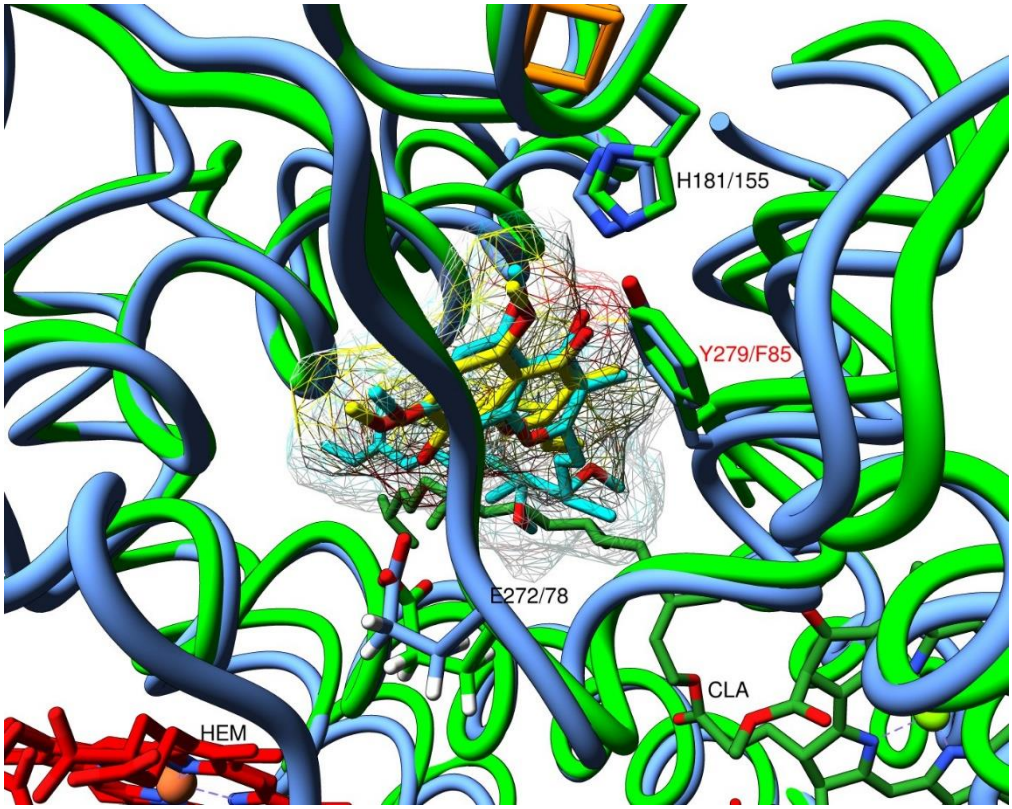

Figure 16. Alignement des structures du complexe bc_1 (bleu) et du complexe b_6f (vert).
CLA, chlorophylle. HEM, hème. Les stigmatellines co-cristallisées avec les deux structures sont colorées en cyan (bc_1) et en jaune (b_6f). Code PDB du complexe bc_1 : 3CX5, code PDB du complexe b_6f :1Q90.

Chapitre I-B. Étude sur des souches « *Plasmodium*-like »

I. Introduction

Bien que la levure soit un bon modèle pour étudier le complexe bc_1 du parasite, les résidus qui sont dans le site Q_o ne sont pas identiques (Figure 1). Des mutants de levure dans lesquels les acides aminés non-conservés du site Q_o ont été remplacés par les équivalents du parasite ont été construits précédemment dans le laboratoire (Vallières, Fisher, and Meunier 2013). Ces mutants appelés « *Plasmodium*-like » ou PF semblent un modèle d'étude du site Q_o de *Plasmodium* plus adapté pour étudier les inhibiteurs et les mutations de résistance.

Ces mutants PF ont été caractérisés par leurs croissances respiratoires, leurs activités du complexe bc_1, leurs sensibilités à différentes inhibiteurs. Le souche PF-Y (PF-11 dans l'article (Vallières, Fisher, and Meunier 2013)) combine 10 changements: C133V, C134L, V135P, Y136W, H141Y, L275F, R283K, M295V, F296L and I299L. La levure, au lieu d'avoir un motif VLPW de 133-136 comme chez les mammifères et le parasite, a CCVY. Ces résidus ne sont pas directement dans le site Q_o mais la séquence $CCVY_{133\text{-}136}$ levure a été néanmoins remplacée par la séquence plus habituelle VLPW (Figure 1 de l'article). Les autres résidus (de la position 141 à la position 299) dans la liste ci-dessus se situent tous dans le site Q_o et sont dans un rayon de 5Å autour de l'atovaquone.

Dans cette étude, j'ai tout d'abord caractérisé l'effet de la mutation de résistance à l'atovaquone Y279S dans la souche PF-Y (cette nouvelle souche s'appelle PF-S). J'ai mesuré la sensibilité des complexes bc_1 des mutants PF-Y et PF-S à l'atovaquone. J'ai observé que le complexe PF-Y est plus sensible à l'atovaquone que l'enzyme contrôle tandis que le complexe PF-S est beaucoup plus résistant à l'atovaquone que le simple mutant Y279S. Les résultats obtenus avec les mutants PF reproduisent donc mieux ce qui a été observé chez le parasite que les résultats obtenus avec les simples mutants.

L'introduction de 10 changements dans le site catalytique Q_o (la souche PF-Y) diminue d'environ 42% d'activité du complexe bc_1. L'introduction d'Y279S dans PF-Y (PF-S) diminue encore l'activité du complexe bc_1 à 34% de l'activité contrôle. Cependant, malgré la diminution d'activité, les souches mutantes devraient pourvoir pousser en milieu respiratoire. En effet, l'étude d'autres mutants du complexe bc_1 a montré qu'il faut descendre en dessous de 25% de l'activité du contrôle pour observer un arrêt de la croissance. Or PF-Y a une croissance très affectée et PF-S ne peut pas pousser en milieu respiratoire. Cette contradiction apparente nous a fait soupçonner que le site Q_o muté fonctionnait mal et que des ions superoxide étaient surproduits lors de la réaction catalytique (*c.f.* « Introduction Générale » D.2.).

L'activité du complexe bc_1 que nous mesurons par spectrophotométrie en utilisant des mitochondries purifiées est l'activité de la réduction du cytochrome *c* en donnant comme substrat, un analogue de l'ubiquinol, le decylubiquinol. Le cytochrome *c* est réduit par les électrons provenant du quinol, via le groupe [2Fe-2S] de l'ISP lors du cycle Q (*c.f.* « Introduction Générale » D.1.). Mais le cytochrome *c* peut aussi être réduit par les ions superoxide (SO) qui seraient produits au site Q_o s'il y a un dysfonctionnement de ce site (Figure 17). Pour estimer la contribution de la réduction par le SO dans la réduction du cytochrome *c*, j'ai mesuré la vitesse de réduction du cytochrome *c* en absence et en présence de superoxide dismutase (SOD) et la catalase, ajoutées au milieu de réaction. Pour le complexe bc_1 de la souche sauvage, la réduction par le SO n'était pas détectable. Il semble donc que l'enzyme ne produit pas de SO lors de

son fonctionnement normal, comme cela avait déjà été observé (Wenz *et al.* 2006). Par contre, pour les mutants PF-Y et PF-S, j'ai observé que environ 30% de la réduction du cytochrome *c* était due au SO. La surproduction de SO par les complexes bc_1 mutants indique que le cycle Q est court-circuité, ce qui résulte en une perte d'énergie et peut expliquer le défaut de croissance.

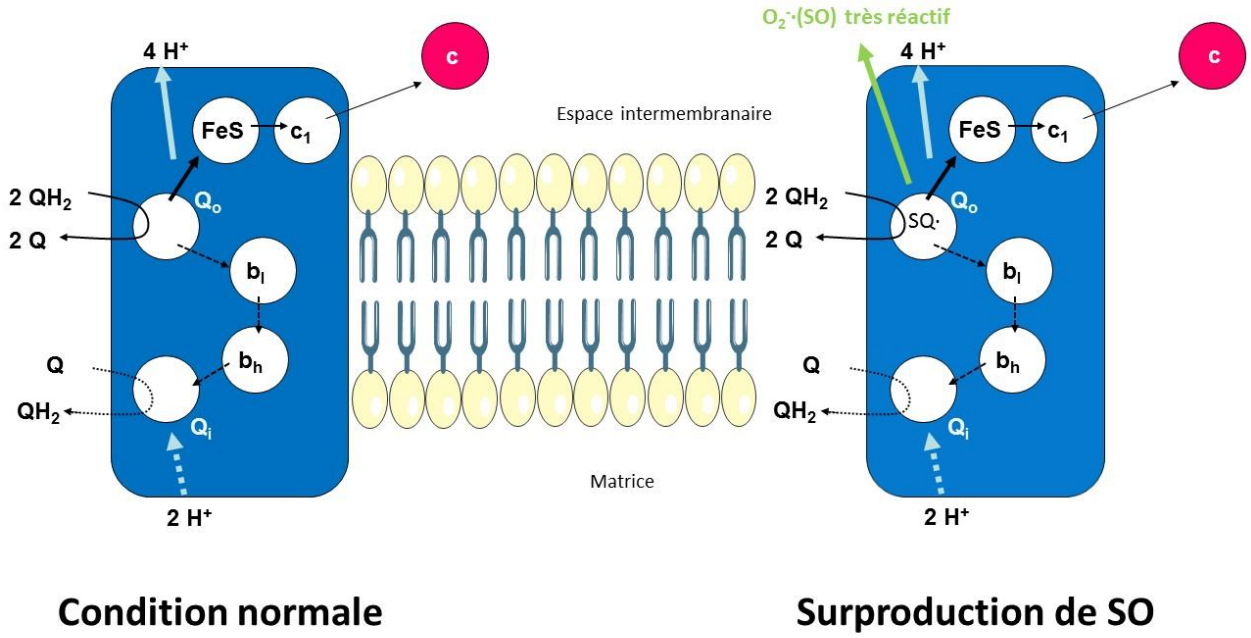

Figure 17. Représentation de la production de SO dans le site Q_o.
Dans la condition normale (gauche), la semiquinone a une durée de vie très réduite et pas ou peu de SO est produit. Si, à cause d'un changement du site Q_o, la semiquinone reste plus longtemps (droite) dans le site Q_o, cette molécule active aura plus d'occasion de réagir avec l'O_2 de l'environnement pour former plus de SO.

Partant du mutant PF-Y à croissance respiratoire faible, nous avons isolé des suppresseurs. Les mutations de suppressions qui restaurent la croissance ont été identifiées. Elles sont localisées dans la région charnière de l'ISP ou dans le cytochrome *b*, en contact avec la région charnière. Comme discuté dans la partie « Introduction Générale - D. Complexe bc_1 », la région charnière de l'ISP joue un rôle important dans le positionnement du groupement globulaire sur le site Q_o et dans la mobilité de ce groupement entre le cytochrome *b* et le cytochrome c_1.

Puisque les mutations de suppressions se situaient dans ou en contact avec la région charnière, nous avons testé s'il est possible de restaurer la croissance respiratoire de PF-Y en remplaçant les résidus de cette région (9 aminoacides) par les équivalents de *P. falciparum*. Nous avons observé que la combinaison du site Q_o type PF avec une région charnière de l'ISP type PF résultait en une meilleure croissance respiratoire. Cette observation est en agrément avec le travail de Weichun Kao *et al.* (Kao and Hunte 2014) qui montre que les partenaires du site Q_o (le substrat, le motif de liaison du site Q_o, et l'ISP) ont co-évolué.

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Interplay between the hinge region of Iron Sulfur Protein and the Q_o site in the *bc*$_1$ complex – Analysis of *Plasmodium*-like mutations in the yeast enzyme.

Zehua Song[a], Jérôme Clain[b], Bogdan I. Iorga[c], Cindy Vallières[a,1], Anaïs Lalève[a], Nicholas Fisher[d], and Brigitte Meunier[a]

[a] Institute for Integrative Biology of the Cell (I2BC), CEA, CNRS, Université Paris-Sud, 91198 Gif-sur-Yvette, France

[b] UMR 216, Faculté de Pharmacie de Paris, Université Paris Descartes, and Institut de Recherche pour le Développement, 75006, Paris, France

[c] Institut de Chimie des Substances Naturelles, CNRS, UPR 2301, Labex LERMIT, 91198 Gif-sur-Yvette, France

[d] Plant Research Laboratory, Michigan State University, East Lansing, MI 48824, USA.

Corresponding authors:

Brigitte Meunier, Institute for Integrative Biology of the Cell (I2BC), CEA, CNRS, Université Paris-Sud, 1 avenue de la Terrasse, 91198 Gif-sur-Yvette, France

Tel 00 33 1 69823156

meunier@cgm.cnrs-gif.fr

Nicholas Fisher, Plant Research Laboratory, Michigan State University, East Lansing, MI 48824, USA

nefisher@msu.edu

[1] Current address: School of Life Sciences, University Park, University of Nottingham, Nottingham, NG7 2RD, UK

Abstract

The respiratory chain bc_1 complex is central to mitochondrial bioenergetics and the target of antiprotozoals. We characterized a modified yeast bc_1 complex that more closely resemble *Plasmodium falciparum* enzyme. The mutant version was generated by replacing ten cytochrome *b* Q_o site residues by *P. falciparum* equivalents. The *Plasmodium*-like changes caused a major dysfunction of the catalytic mechanism of the bc_1 complex resulting in superoxide overproduction and respiratory growth defect. The defect was corrected by substitution of the conserved residue Y279 by a phenylalanine, or by mutations in or in the vicinity of the hinge domain of the iron-sulphur protein. It thus appears that the substitution Y279F or the modification of the iron-sulphur protein hinge region prevented side-reactions. Interestingly, *P. falciparum* – and all the apicomplexan- contains an unusual hinge region. We replaced the yeast hinge region by the *Plasmodium* version and combined it with the *Plasmodium*-like version of the Q_o site. This combination restored the respiratory growth competence. It could be suggested that, in the apicomplexan, the hinge region and the cytochrome *b* Q_o site have co-evolved to maintain catalytic efficiency of the bc_1 complex Q_o site.

Keywords: respiratory complex III, malaria parasite, yeast model, superoxide production.

Short title: *Plasmodium*-like version of yeast bc_1 complex

Abbreviations used: Q_o, quinol oxidation site; Q_i, quinone reduction site; Cyt *b*/*c*, cytochrome *b*/*c*; ISP, Iron-Sulfur Protein; SO, superoxide ; SOD, superoxide dismutase; OD, optical density; DQ, decylubiquinol; k_{cat}, catalytic activity reported on bc_1 complex concentration; IC_{50}, inhibitor concentration required to obtain 50% inhibition

1 INTRODUCTION

The mitochondrial respiratory chain complex III or bc_1 complex is central to mitochondrial bioenergetics and the target of antiprotozoals, such as atovaquone, and fungicide drugs used to control human and plant pathogens. It is a multimeric enzyme. Three subunits form the catalytic core contain the redox-active groups haems and FeS, namely two cytochromes *b* and c_1 and the iron-sulphur protein (ISP). The structures of several complexes have been solved. They share the same catalytic core. The central and largest subunit is cytochrome *b*, a predominantly hydrophobic protein consisting of eight transmembrane helices. Cytochrome *b* is mitochondrially encoded in all eukaryotes while the other subunits are nuclearly encoded.

The bc_1 complex catalyses the transfer of electrons from ubiquinol to cytochrome *c* and couples this electron transfer to the vectorial translocation of protons across the inner mitochondrial membrane, contributing to the protonmotive force used for ATP synthesis by the ATP synthase. The catalytic mechanism, called the Q-cycle (see for instance, [1,2]), bifurcates electron flow across high- and low-potential electron transfer chains within the bc_1 complex, and requires two distinct quinone-binding sites (Q_o, quinol oxidation site, and Q_i, quinone reduction site), which are located on opposite sides of the membrane and linked by a transmembrane electron-transfer pathway, reducing one molecule of ubiquinone per two molecules of ubiquinol oxidised. Cytochrome *b* provides both the Q_o and Q_i pockets and the (low potential) transmembrane electron pathway (*via* haems b_L and b_H). The high potential chain from Q_o to cytochrome *c* is formed by the ISP and cytochrome c_1. An unusual property of the catalytic cycle of the bc_1 complex is the role of the C-terminal, extrinsic domain of the ISP (the [2Fe-2S]-containing 'headgroup') which acts as a tethered, mobile substrate, shuttling electrons from Q_o-bound ubiquinol within cytochrome *b* to the haem of cytochrome c_1, alternately docking with these protein partners [3].

As a one-electron carrier, this large-scale movement of the ISP is likely to form one mechanism to assist in preventing energetically wasteful electron transferring bypass reactions, such as double reduction of the high potential chain [4]. Movement of the ISP headgroup is thought to be facilitated by relaxation of a short 3_{10} helical and turn region of the structure known as the 'hinge' or 'linker', which connects the C-terminal domain of the ISP to its helical transmembrane anchor [5,6] (for a review, see [7]).

The Q_o site is a relatively large domain formed from components encompassing amino acid residues 120-150 and 260-300 of the cytochrome *b*. On the basis of structural and mutational analysis, Q_o inhibitors can be separated into two classes by their mode of binding ([8,9]). For instance, azoxystrobin binds in the so-called 'haem b_L proximal' domain of the Q_o site whereas stigmatellin and atovaquone bind in the 'haem b_L distal' domain, occupying the region of the Q_o site close to the interface with the ISP. Several atomic structures of the yeast (*Saccharomyces cerevisiae*) bc_1 complex with bound Q_o site inhibitors are available. Of particular interest is the structure of the yeast complex with the anti-malarial drug atovaquone bound [10].

To date, atovaquone (in combination with proguanil) is the sole bc_1 complex inhibitor used as antimalarial drug in treatment and causal prophylaxis. The compound is also active against *Pneumocystis jirovecii*, *Toxoplasma gondii*, *Babesia* sp. and other pathogens. Unfortunately, atovaquone-resistant parasite populations can rapidly emerge during therapy, leading to treatment failure (see for instance, [11,12]). In the human malaria parasite *Plasmodium falciparum*, the resistance is caused by the mutation of cytochrome *b* residue Y268 (Y279 yeast numbering) in the drug target. New drugs are needed and different compounds are currently being studied (see for instance, [14-16]). Whilst the necessary gold standard, enzymological and physicochemical investigations of the parasite enzyme required for the development and study of new drugs are technically challenging due to the difficulties in preparing mitochondrial fractions from this organism. Easy-to-use models that mimic the parasite enzyme facilitate the analysis of new drugs and the study of the development of resistance mutations. Yeast provides a useful surrogate model as cytochrome *b* amino acid sequences, in particular at Q_o and Q_i sites, are highly conserved between organisms. In addition, yeast is amenable to mitochondrial transformation and site-directed modification of mtDNA genes. The yeast Q_o and Q_i sites can thus be reconstructed to mimic the parasite active domains. In a previous study, we generated a series of yeast mutants by replacing yeast Q_o site residues by *P. falciparum* or human residues to explore the variations in the Q_o site that could explain the differential sensitivity to atovaquone between these species [17]. Here we investigated the effect of inter-species variations on the finely tuned mechanism of the enzyme.

We characterized a modified yeast bc_1 complex that more closely resembles that of the human malaria parasite *P. falciparum*. The mutant version was generated by replacing ten cytochrome *b* Q_o site residues by *P. falciparum* equivalents. We measured the effect of multiple changes on the respiratory growth competence, on the bc_1 complex activity and its sensitivity to inhibitors and on superoxide production. The *Plasmodium*-like changes caused a severe dysfunction of the complex resulting in increased superoxide production. We then investigated ways to restore the respiratory competence.

2 Materials and Methods

2.1 Materials and growth media

Equine cytochrome *c*, decylubiquinone, azoxystrobin, antimycin, atovaquone, superoxide dismutase, and catalase were obtained from Sigma Aldrich. The following media were used for the growth of yeast: YPD (1% yeast extract, 2% peptone and 3% glucose), YPGal (1% yeast extract, 2% peptone and 2% galactose), YPeth (1% yeast extract, 2% peptone and 3% ethanol) and YPG (1% yeast extract, 2% peptone and 2% glycerol).

2.2 Yeast strains and plasmids

The cytochrome *b* mutants were generated by side-directed mutagenesis and mitochondrial transformation [17,18]. They have identical nuclear and mitochondrial genomes with the exception of the mutations introduced in the cytochrome *b* gene.
The strains carrying a deletion of *RIP1*, encoding the ISP, were derived from JPJ1 [19] and were generated by cytoduction *i.e.* by transferring the mitochondrial genome from a donor strain into the mtDNA-less (rho^0) derivative of a recipient strain.
The centromeric plasmid carrying wild-type (wt) *RIP1* under the control of its own promoter was provided by Prof. B. Trumpower [19].

2.3 Measurement of decylubiquinol-cytochrome *c* reductase activity

Yeast mitochondria were prepared as in [20]. Briefly, yeast grown in YPGal medium were harvested at mid-log phase. Protoplasts were obtained by enzymatic digestion of the cell wall using zymolyase in an osmotic protection buffer. Mitochondria were then prepared by differential centrifugation following osmotic shock of the protoplasts. Mitochondrial samples were aliquoted and stored at -80 °C. Concentration of bc_1 complex in the mitochondrial samples was determined from dithionite-reduced optical spectra, using ε=28.5 mM^{-1} cm^{-1} at 562 nm *minus* 575 nm. Decylubiquinol-cytochrome *c* reductase activities were determined at room temperature by measuring the reduction of cytochrome *c* (final concentration of 20 µM) at 550 nm *versus* 540 nm over 1-min time-course in 10 mM potassium phosphate pH 7, 0.01% (w/v) lauryl-maltoside and 1 mM KCN. Mitochondria were added to obtain a final concentration of 5-30 nM bc_1 complex. Activity was initiated by the addition of decylubiquinol, a synthetic analog of ubiquinol (final concentrations of 2.5-60 µM). Initial rates were measured as a function of the decylubiquinol concentration. Each measurement was repeated three to five times and the values obtained were averaged. K_M values were estimated from the plots of cytochrome *c* reduction rates *vs* decylubiquinol concentrations. k_{cat} were determined as the cytochrome *c* reduction rate per bc_1 complex. The mid-point inhibition concentrations (IC_{50}) were determined by inhibitor titration.

3. RESULTS

3. 1. A *Plasmodium*-like version of the Q_o site in yeast bc_1 complex

We have generated a modified version of yeast bc_1 complex with a Q_o site that more closely resembles the parasite Q_o site by replacing ten residues by *P. falciparum* equivalents (Figs 1 and 2) [17]. These residues are conserved in all the available *Plasmodium* sequences.

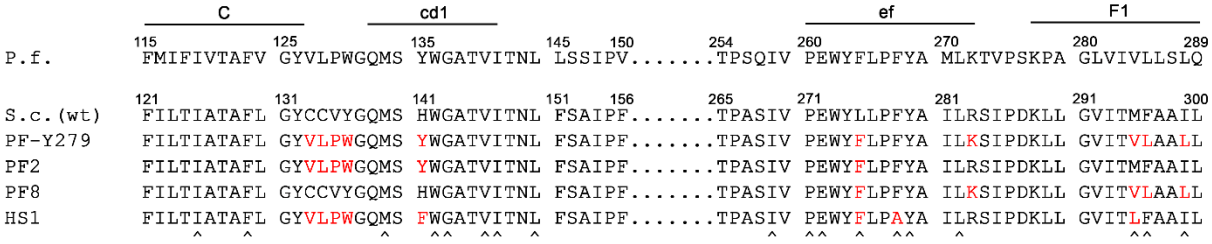

Fig.1. Q_o site sequences and aminoacid substitutions in the mutants. Sequence comparison of the Q_o site domain between *P. falciparum* (P.f) and yeast (S.c), wt and mutants PF-Y279, PF2, PF8 and HS1 is shown. The aminoacid substitutions in the *Plasmodium*-like (PF) and human-like (HS1) mutants are marked in color. The arrow heads indicate the residues in close contact with bound atovaquone [10]. C, cd1, ef and F are structural elements within the Q_o domain.

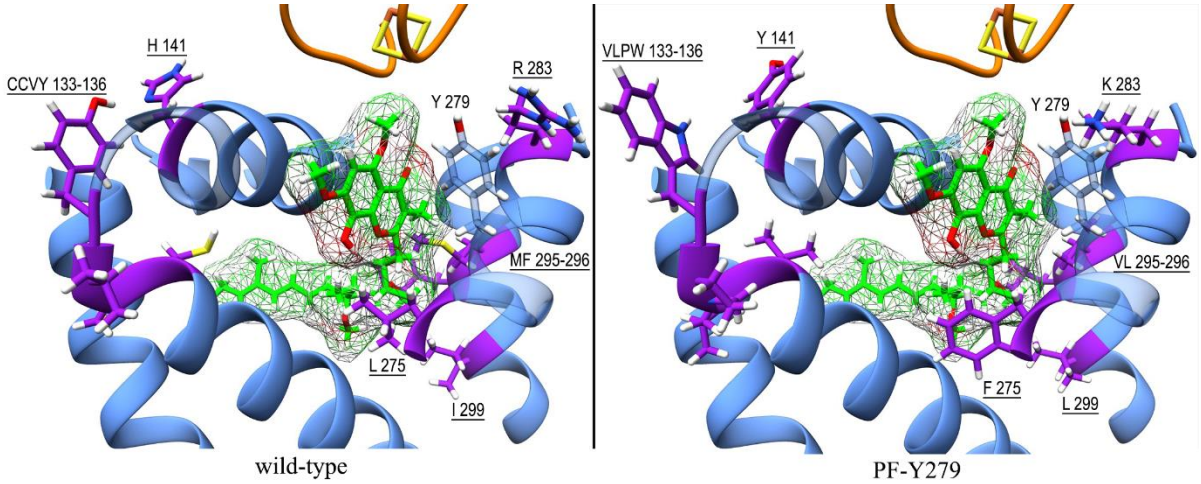

Fig.2. Structure of the Q_o site and location of the mutations. Cytochrome *b* is in blue: ISP in orange with the Fe-S cluster in yellow and sigmatellin in green. The left hand side panel shows the wt residues. The right hand side panel shows the *Plasmodium*-like substitutions introduced in mutant PF-Y279. The ten modified residues are underlined and high lightened in violet. The conserved residue Y279, key for atovaquone binding is in grey. Hydrogen atom is in white, oxygen in red, nitrogen in dark blue and sulphur in yellow. The structure of the yeast bc1 complex was drawn using the coordinates of PDB ID: 3CX5. *In silico* mutations were introduced using CHIMERA, which was also used for generating the molecular modeling images.

The resulting *Plasmodium*-like bc_1 complex (named PF-Y279) was sensitive to atovaquone, a leading antimalarial drug used (in combination with proguanil) against *Plasmodium falciparum*.

We then introduced in PF-Y279 the most prevalent atovaquone-resistance mutation found in *P. falciparum* that associates with malaria treatment failure, namely Y279S. The mutant PF-Y279S was highly resistant to atovaquone (> 250-fold compared to PF-Y279) (Table 1). We also monitored the sensitivity of the wt and mutant enzymes to antimycin and azoxystrobin, two well-known bc_1 complex inhibitors that bind at different target sites: antimycin targets the Q_i site while azoxystrobin binds in the Q_o site but in position different to atovaquone, (*i.e.* closer to haem b_L) [9]. Control and mutants showed similar sensitivity to these inhibitors.

Table 1: bc_1 complex activity and sensitivity to inhibitors. Decylubiquinol cytochrome *c* reduction activities were monitored as described in Materials and Methods and reported as the cytochrome *c* reduction rates per bc_1 complex as a function of decylubiquinol concentration. k_{cat} (at 20 □M decylubiquinol) and K_M values were estimated from the plots presented in Fig.4. IC_{50}/bc_1, inhibitor concentrations required to reach 50% inhibition were obtained by inhibitor titration and reported on bc_1 complex concentration. DQ, decylubiquinol.

Strains	Decylubiquinol cytochrome *c* reductase activity					
	k_{cat}	K_M	k_{cat} / K_M	IC_{50}/bc_1		
	s^{-1}	DQ μM		atovaquone	azoxystrobin	antimycin
Y279 (wt)	53 ± 1.4	3.1	17	4 ± 0.6	12 ± 1.2	0.3 ± 0.01
PF-Y279	22.7± 0.8	7.9	3.9	2 ± 0.1	12 ± 0.4	0.3 ± 0.01
PF-Y279S	18.2± 0.5	4.3	4.2	>500	7 ± 0.9	0.2 ± 0.02
PF-Y279F	54 ± 0.9	6.4	8.9	3 ± 0.1	5 ± 0.3	0.4 ± 0.03
PF-Y279+H53Y	56.5± 3.2	6	9.3	2 ± 0.1	29 ± 2.1	0.8 ± 0.15

3.2. Assessment of Q_o site dysfunction

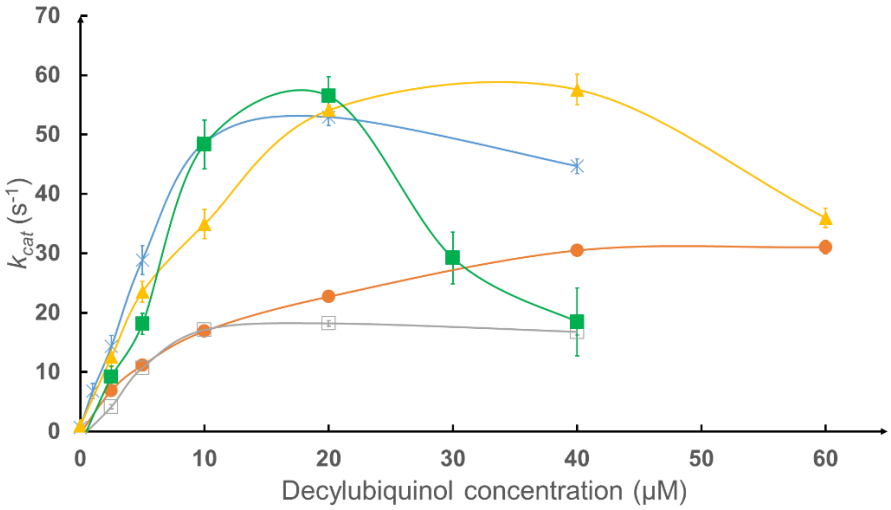

Fig.3 Decylubiquinol cytochrome *c* activity. Decylubiquinol cytochrome *c* reduction activities were monitored as described in Materials and Methods and reported as the cytochrome *c* reduction rates per bc_1 complex as a function of decylubiquinol concentration. Each measurement was repeated three to five times and the values obtained were averaged. The error-bars represent standard deviations.
Wild-type (blue line); mutants PF-Y279 (orange) and PF-Y279S (grey); suppressors PF-Y279F (yellow) and PF-Y279+H53Y (green).

As shown in Fig.3 and Table 1, the mutated bc_1 complexes were apparently active despite the multiple modifications of the Q_o site. Cytochrome *c* reduction activities of PF-Y279 and PF-Y279S (at 20 μM decylubiquinol) were respectively 43 and 34 % of the wt rate. As judged by the K_M for decylubiquinol, the bc_1 complex with the PF-like Q_o site had a lower affinity for the substrate than the native enzyme (7.9 μM for PF-Y279 as compared to 3.1 μM for wt). It was slightly lower for PF-Y279S (4.3 μM). Thus the ratio k_{cat} / K_M, a second-order rate constant that gives an estimate of the catalytic efficiency, was decreased from 17 for the wt to 3.9 and 4.2 for PF-Y279 and PF-Y279S, respectively.

The respiratory growths of PF-Y279 and PF-Y279S mutants were severely affected. As presented in Fig.4, PF-Y279 showed a very weak respiratory growth. PF-Y279S was unable to grow.

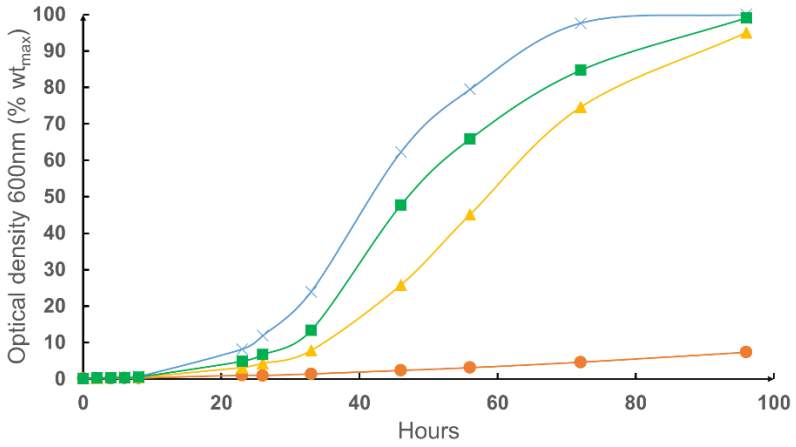

Fig.4 Respiratory growth. wt and mutants were pre-grown in YPeth medium, then inoculated in fresh YPeth medium to obtain an OD_{600} of 0.1. The cultures were incubated with vigorous agitation at 28 °C. Growth measured as OD at 600 nm was monitored for four days.
Wild-type (blue line); mutant PF-Y279 (orange); suppressors PF-Y279F (yellow) and PF-Y279+H53Y (green).

We have previously observed that the loss of 60% of the bc_1 complex activity had only a moderate effect on respiratory growth [21]. The cut-off value to sustain respiratory growth for our strains and conditions was between 15 and 20% of wt enzyme activity (unpublished data).

It could thus be expected that mutant bc_1 complexes that showed 43 % and 34% of wt activity would support a good respiratory growth.

It seemed therefore that the *Plasmodium*-like modifications of the Q_o site caused a major dysfunction of the enzyme that could not support the expected growth.

3.3. Superoxide over-production

Dysfunction of the Q_o site (manifested as electron leak from semiquinone to oxygen) may result in the overproduction of superoxide (SO). We thus monitored SO production as the superoxide dismutase (SOD)-sensitive rate of cytochrome *c* reduction. The difference between the reduction rate in the absence and the presence of added SOD gives the contribution of the cytochrome *c* reduction by SO to the overall cytochrome *c* reductase activity (Fig.5).

Wild-type bc_1 complex did not produce SO, as previously shown [22,23]. By contrast, 25% of the DQ-cytochrome *c* reduction activity by PF-Y279 and PF-Y279S was SOD sensitive. Thus these modified forms of bc_1 complex produced a significant amount of SO.

The overproduction of SO is a clear signifier of Q-cycle dysfunction, suggestive of increased semiquinone occupancy or otherwise energetically wasteful electron transfer reactions, with a significant loss of energy [4]. That major dysfunction of the catalytic mechanism could explain the defective respiratory growth.

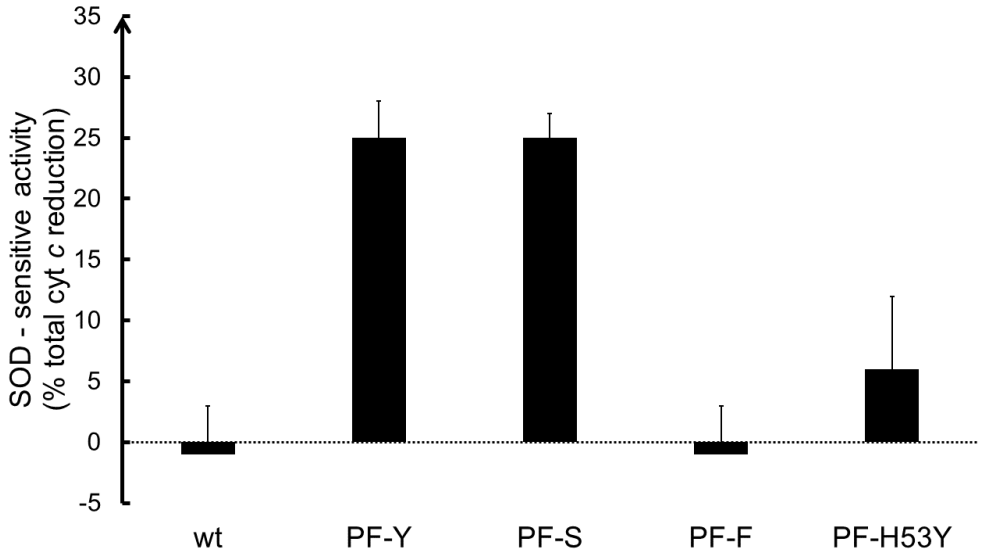

Fig.5 Superoxide production in wild-type and mutants. PF-Y, PF-Y279; PF-S, PF-Y279S; PF-F, PF-Y279F and PF-H53Y, PF-Y279+H53Y. Cytochrome *c* reduction rates were recorded in absence and in presence of SOD and catalase, both at 225 units/mL, using 20 □M of decylubiquinol. The data are presented as percentage of SOD-sensitive cytochrome *c* reduction. The assays were performed as described in Materials and Methods, with slight modifications made to optimize the SOD and catalase activities: decylubiquinol-cytochrome *c* reduction was assayed in potassium phosphate buffer 50 mM pH7 with 50 □M cytochrome *c* and 10 □M KCN. Each measurement was repeated at least three times and averaged.

In addition, the SO produced by the mutated enzymes might contribute to the growth defect by damaging ROS-sensitive components. We checked whether the respiratory growth competence of PF-Y279 could be improved by the over-expression of *SOD2* (encoding the mitochondrial matrix superoxide dismutase) or *SOD1* (encoding the enzyme localised in the cytoplasm and the mitochondrial intermembrane space). No improvement was observed (not shown).

3.4. Q_o site dysfunction caused by the combined *Plasmodium*-like mutations

The *Plasmodium*-like Q_o site harboured ten amino-acid substitutions of the yeast Q_o site residues: $CCVY_{133\text{-}136}$VLPW, H141Y, L275F, R283K, $MF_{295\text{-}296}$VL and I299L. We investigated whether the mutated residue(s) causing the inefficient catalytic activity could be pinpointed. To that end, we analysed three mutants carrying different combination of mutations (Fig.1). PF2 and PF8 harboured respectively five and six of the *Plasmodium*-like mutations: $CCVY_{133\text{-}136}$VLPW, H141Y and L275F, in PF2; L275F, R283K, $MF_{295\text{-}296}$VL and I299L, in PF8. In HS1, eight residues have been replaced by the human equivalents: $CCVY_{133\text{-}136}$VLPY, H141F, L275F, F278A and M295L (Fig.1).

The respiratory growth and DQ-cytochrome *c* reductase activity of these mutants were tested. They showed wt or close to wt growth and activities (data not shown). It seems therefore that the stringent growth defect of PF-Y279 is caused by the combination of the *Plasmodium*-like changes.

3.5. Suppressors

In order to get more information on the dysfunction of the *Plasmodium*-like bc_1 complex, suppressors - respiratory growth competent cells- were selected from the mutants PF-Y279 and PF-Y279S. For that end, the mutants were subcloned. Several subclones were grown in YPD, and then incubated on respiratory medium (YPG). Respiratory competent clones appeared after a two- or three-week incubation. Several independent suppressors (each from different subclones) were then analyzed. The suppressor mutations were identified as described in [24].

From PF-Y279, ten suppressors with a vigorous respiratory growth were studied. Five harboured a secondary mutation in cytochrome *b*: H53Y (obtained twice), H53D (obtained twice) and P174S. In the other five suppressors, the compensating mutation was located in the hinge region of the ISP: A86P, D87G, V88F, L89F and A90T. Interestingly, residues H53 and P174 are close to the hinge region of the ISP.

Starting from PF-Y279S, suppressors with a very weak respiratory growth were obtained. The compensating mutations were also located in the hinge region of the ISP: A86P, D87N, V88A/F/D, L89F and A90V/D. Interestingly, one suppressor restoring a vigorous respiratory growth was obtained from PF-Y279S. The mutation replaced Y279S by Y279F. The resulting strain was named PF-Y279F.

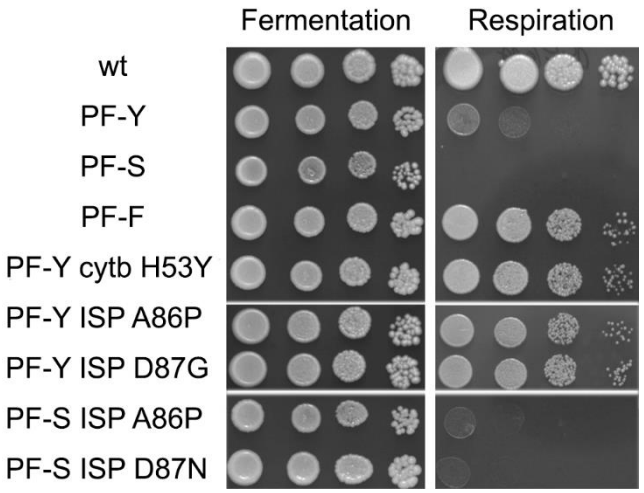

Fig.6 Respiratory growth competence of mutants and suppressors.
Serial dilutions in water of cells pre-grown on glucose plates were spotted on plates containing either fermentation medium (YPD) or respiration medium (YPG) and incubated for three days at 28 °C. PF-Y279 (PF-Y); PF-Y279S (PF-S); PF-Y279F (PF-F); suppressors that combined the Q_o site mutations of PF-Y279 with a suppressor mutation in cytochrome *b* (PF-Y cyt*b* H53Y) or in ISP (PF-Y ISP A86P and PF-Y ISP D87G); suppressors that combined the Q_o site mutations of PF-Y279S with a suppressor mutation in ISP (PF-S ISP A86P and PF-S ISP D87N).

Fig.6 shows the growth capacity of mutants PF-Y279 and PF-Y279S, of the suppressor PF-Y279F, of three suppressors issued from PF-Y279 that harbour a compensatory mutation in cytochrome *b* (H53Y) or in ISP (A86P and D87G) and of two suppressors issued from PF-Y279S with a suppressor mutation in ISP (A86P and D87N). On fermentation medium (YPD), mutants and suppressors grew equally well as the cells relied on fermentation to proliferate. On respiration medium (YPG), the impaired growth of PF-Y279 was clearly observed while PF-Y279S was unable to grow. The combination of PF-Y279S with the mutation in ISP resulted in a very weak respiratory growth while a vigorous respiratory growth competence was restored in the other suppressors.

PF-Y279F and PF-Y279-H53Y were then analysed in more details. As shown in Table 1 and Fig.3, the additional substitution Y279F or H53Y in the *Plasmodium*-like Q_o site PF-Y279 resulted in an enzyme with a wt turnover at 20 □ M decylubiquinol but with a higher K_M.

Increasing the decylubiquinol concentration above 20 □ M resulted in severe loss of the activity of PF-Y279-H53Y (Fig.3). At 40 µM, its k_{cat} was decreased to 18.5 s^{-1}, thus to 32% of the rate at 20 □ M decylubiquinol. A decrease in activity with increasing substrate concentration was also observed with PF-Y279F but to a lesser extent, the k_{cat} decreasing from 58 s^{-1} at 40 µM substrate to 36 s^{-1} at 60 µM substrate. This behaviour was not observed with PF-Y279 and PF-Y279S. The wt enzyme displayed a small (15%) loss of activity at 40 µM decylubiquinol. The sharp loss of activity observed with PF-Y279-H53Y could

be caused by the weakly chaotropic nature of decyubiquinol that could impair the stability of the mutant enzyme. We have previously observed a similar behaviour with a cytochrome *b* mutant carrying a substitution of residue G167 (G167E), which is located in the cd2 loop of cytochrome *b* in close contact with the ISP hinge region [25].

Interestingly, the suppressor mutations significantly decreased the production of SO (Fig.5). They thus seem to restore a Q-cycle with little by-pass reactions.

The respiratory growth competence was not fully restored as PF-Y279F and PF-Y279-H53Y showed a slower growth rate than wt (Fig.4). This could be explained, at least in part, by the lower catalytic efficiency estimated by the ratio k_{cat} /K_M.

3.6. Yeast *vs Plasmodium falciparum* Q_o site

The introduction of *Plasmodium*-like mutations in yeast Q_o site altered the catalytic activity of the bc_1 complex, leading to an increase in the rate of energetically wasteful electron transfer bypass reactions (as assessed by superoxide production) and by consequence to a decreased growth competence.

As we found suppressor mutations in the ISP hinge region, we tested whether *Plasmodium*-like modifications of the ISP hinge region could improve the activity of the *Plasmodium*-like Q_o site in the yeast enzyme and thus restore the respiratory growth competence of PF-Y279.

The ISP hinge region is formed of 12 residues (residues 81 – 92 in yeast sequence), well conserved between yeast and human, but it largely differs in *P. falciparum* where only two residues are conserved (Fig.7). That unusual sequence in the parasite, found also in other *Apicomplexa*, might be required for the optimal activity of the plasmodial Q_o site.

We generated two new versions of the protein by replacing eight (ISP1) and ten (ISP2) yeast residues by the *P. falciparum* equivalents (Fig.7).

The changes were introduced by site-directed mutagenesis in the plasmid-borne *RIP1* gene, encoding yeast ISP [19]. The plasmids carrying wt or mutated *RIP1* were then used to transform strains that carry both a deletion of genomic *RIP1* and the wt or the mutated cytochrome *b* (PF-Y279). The respiratory growth competence of the mutant strains was then tested.

As shown in Fig.7, the combination of the *Plasmodium*-like version of the Q_o site (PF-Y279) with a modified *Plasmodium*-like version of the ISP hinge region (ISP1) significantly improved the respiratory growth competence. The ISP1 modification was also well accommodated in the bc_1 complex with the native Q_o site. By contrast, the combination of ISP2 with the native Q_o site resulted in a severe decrease of respiratory growth while that same mutation ISP2 slightly improved the growth of PF-Y279. ISP2 version differs from ISP1 by two additional changes, M91G and A92G, which seem to have a stringent effect.

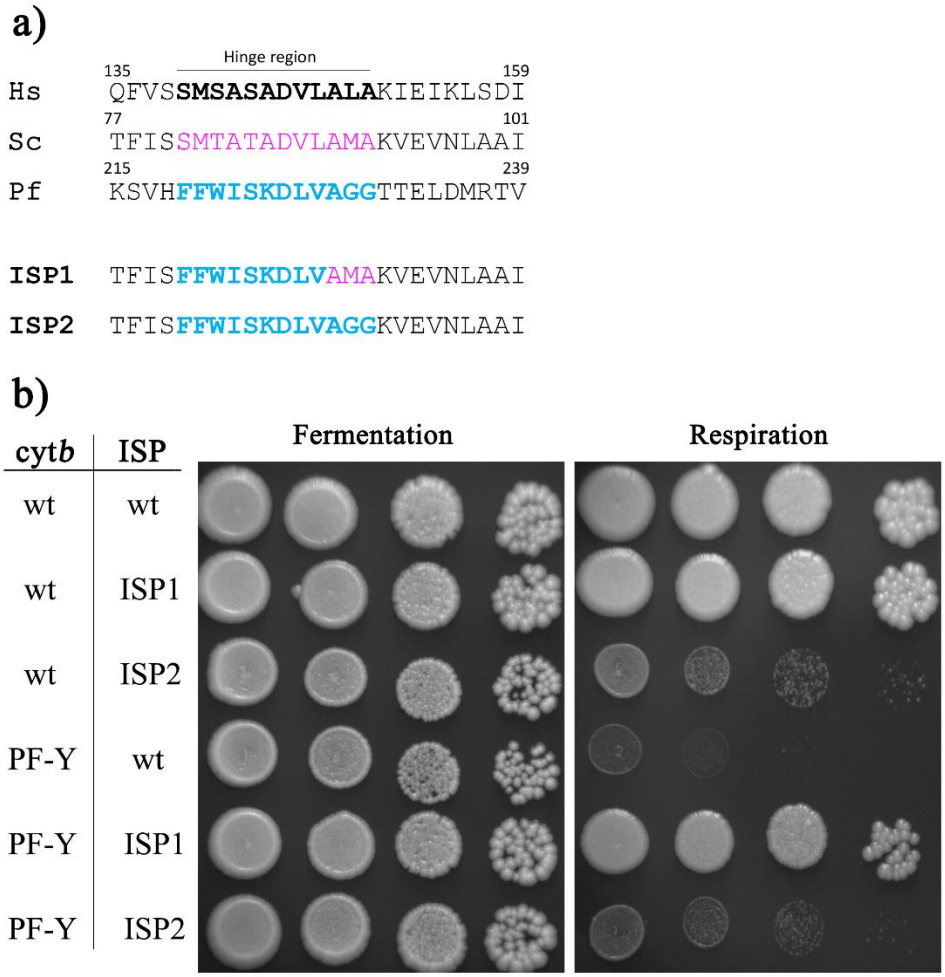

Fig.7 Respiratory growth of mutants combining Q_o- and ISP- *Plasmodium*-like modifications
a) Sequence of the ISP hinge region of human (Hs), yeast (Sc), *P. falciparum* (Pf), and yeast mutants with a modified version of the hinge region (ISP1 and ISP2) where yeast residues (magenta) have been replaced by the parasite residues (blue).
b) Growth competence of yeast strains that combined a wt Q_o site or a PF-Y279 version (PF-Y) with wt or modified ISP hinge region (ISP1 and ISP2).

We then tested whether the *Plasmodium*-like modifications of the ISP hinge domain could also restore the respiratory growth competence of the mutant PF-Y279S. The mutant strain was thus transformed by the plasmids containing the mutated ISP, ISP1 and ISP2 and the growth competence of the resulting strains was checked. The introduction of ISP1 resulted in a very weak improvement of the respiratory growth, very similar to that observed for the ISP-located suppressors selected from PF-Y279S (as shown in Fig.6). ISP2 had no effect on the growth defect of the mutant.

4. Discussion

4.1. Fixing the impaired Q_o site

The substitution of ten *Plasmodium*-like residues in yeast Q_o site resulted in a bc_1 complex with altered properties, exhibiting decreased steady-state rates of substrate (decylubiquinol) oxidation and affinity. Most importantly, the production of SO was significantly increased. It may be hypothesized that, in the mutant strains, the structure of the Q_o site was perturbed and that the interaction of the ISP headgroup with cytochrome *b* was altered with respect to wt. Consequently, we suggest that the observed increase

in SO production was due to a mechanistic dysfunction at Q_o, resulting in an increased semiquinone occupancy or an increase in the probability of reaction with oxygen by kinetic or thermodynamic means.

Further modifications of the site restored nearly full activity: replacement of Y279 by a phenylalanine or changes in the ISP hinge region or in the cytochrome *b* region in close contact with the ISP hinge region.

A tyrosine is highly conserved at position 279. The residue is likely to be important for substrate positioning at Q_o [26] and is key for atovaquone binding [10]. The mutation of Y279 leads to resistance to the antimalarial agent atovaquone in *P. falciparum* and in mouse malaria models [12,13,27].

We found previously that in the native yeast Q_o site, Y279 could be replaced by a phenylalanine without loss of activity [28]. Here we observed that the substitution of Y279 by a phenylalanine in the *Plasmodium*-like Q_o site (PF-Y279F) restored the bc_1 complex turnover to wt level and prevented the production of SO.

It has been suggested that Y279 is mechanistically important for the correct functioning Q_o site chemistry, with the sidechain of the residue acting as a 'trapdoor' that gates substrate accessibility to the distal region of Q_o. The positioning of the residue is proposed to be governed by displacement of the PEWY loop (the 'PEWY seesaw') and the docking of the ISP headgroup at the Q_o site [29,30]. This proposed 'trapdoor' mechanism is likely to have a role in preventing side-reactions and SO production by minimising semiquinone occupancy at Q_o site. It may be postulated that a phenylalanine, having perhaps more degrees of freedom of movement due to the lack of a potentially hydrogen-bonding hydroxyl moiety, is a more efficient trapdoor for the *Plasmodium*-like Q_o site. It should be noted that in *Plasmodium* sp., a tyrosine is conserved at position 279 (268 in *P. falciparum* sequence).

If the "trapdoor" is impaired in PF-Y279, re-adjustment of the ISP movement or docking could compensate the defect, as the respiratory growth competence of the mutant could be restored by modification of the hinge region of the ISP, namely mutations of ISP residues 86 to 90 and mutations of cytochrome *b* residues H53 and P174 in the vicinity of the ISP hinge region. The bc_1 complex of the suppressor PF-Y279-H53Y showed a near wt turnover with little SO production. It is likely that the other suppressors have the same effect. Thus, the modification of the ISP hinge region seems to prevent the production of SO by the altered Q_o site.

We suggest that the modification of the hinge region results in a more favourable interaction of the ISP on the mutated Q_o site facilitating productive electron transfer and/or restricting accessibility of oxygen to the semiquinone (or otherwise lessening the probability of the reaction between semiquinone and oxygen). It is important to note that the progenitor of SO at Q_o site, semiquinone, is only formed after oxidation of Q_o-bound quinol by the [2Fe-2S] cluster of the ISP (or re-reduction of quinone by reduced haem b_L). Hence, impaired docking of the ISP at the Q_o site leading to the formation of the initial encounter complex between cytochrome *b*, quinol and the ISP, is in itself insufficient to explain increased rates of SO production in the mutant strains described in this study. Such impaired docking would need to increase the accessibility of the site to oxygen or increases the degree of semiquinone occupancy (or, alternatively, reactivity) after the first electron transfer reaction at the Q_o site, increasing the likelihood of oxygen reduction. This may occur if diffusion of the semiquinone intermediate within Q_o towards its productive reaction partner ferrihaem b_L is impeded in some way [8,29], or alternatively by favouring the reduction of product quinone bound within the proximal region of Q_o by ferrihaem b_L, regenerating semiquinone [31].

4.2. Hinge region suppressor hotspot

The hinge region of the ISP (residues 81-92 in yeast) links the transmembrane anchor (residues 51-80) to the extrinsic mobile domain (residues 93-215). Residues 81-86 are stabilized by hydrogen bonds and hydrophobic interactions. A helical turn is formed by residues 86-88 while residues 87-91 are

stretched out. At the end of the hinge region, residue 92 is stabilised by a hydrogen bond with ISP W111 [32]. The flexibility of this hinge region facilitates the movement of the headgroup. It was shown that insertion or deletion of residues, in that domain, had significant effect on the catalytic activity [6,32].

We observed that the severe respiratory defect caused by Q_o site mutations A144F, S152P and G291D [24,33], or by multiple changes (this work) could, at least partly, be alleviated by mutations in the ISP hinge region. The compensatory mutations were found in the stretch from residue 85 to residue 92: T85A/G/I/S, A86L/P/R, D87G, V88A/D/F/G, L89F, A90D/G/S/T/V, A92D/G/T. A suppressor mutation at residue 93, K93E, immediately downstream of the hinge region was also observed. By contrast, no compensatory mutation was found at residue M91 or residues 81-84. It thus seems that the stretch $T_{85}ADVLA_{90}$ (with A92) is a suppressor mutation hotspot.

Suppressor mutations in cytochrome *b* were also found, H53D/Y and P174S. The residues are located in the close vicinity of the hinge region. Note that H53 is close to the hinge region of the ISP that reacts with the other monomer.

We previously studied the effect of the cytochrome *b* mutation G167E, in the cd2 loop, near P174 and in contact with the ISP hinge region. The replacement of G167 with glutamate severely impaired the enzyme activity and stability. We hypothesized that the mutation affected the structure of the hinge region, resulting in a hindered movement and in enzyme instability. This indicated that interfacial protein-protein interactions between the hinge region of ISP and the extramembranous cd2 helix of cytochrome *b* were important to maintain the structure of the hinge region, and by consequence the movement of the headgroup and the integrity of the enzyme [25].

The suppressor mutations that restore the function of PF-Y279, located in the hinge region or in the cytochrome *b* cd2 helix in contact with the hinge region could modify the structure/ propriety of the hinge region, resulting in an improved interaction between the Q_o site and the ISP.

4.3. Adjustment of the ISP hinge region and the Q_o site in the *Plasmodium* enzyme

Most interestingly, replacement of the residues of the hinge region by the *Plasmodium* equivalents also resulted in a restored respiratory growth of PF-Y279.

The hinge region of the *P. falciparum* ISP has a non-canonical sequence in the turn at the C-terminus of the transmembrane helix and the short 3_{10} helical component (residues 81-82 and 86-89 respectively, yeast sequence numbering). These peculiarities are also observed in other *Apicomplexa*. The presence of two aromatic residues at positions 81-82 (yeast sequence) in the hinge region of *Apicomplexa* and replacement of A90 by lysine in the 3_{10} helix might be expected to subtly alter the structure or mobility of the linker, perhaps extending the 3_{10} helical component, or favouring the formation of α-helical structure in this region. These changes may assist the movement and correct positioning of the ISP headgroup on the Apicomplexan Q_o site for efficient choreography of electron transfer at Q_o site, whilst minimising the probability of unwanted side reactions. It is thus likely that the ISP hinge region and Q_o site of cytochrome *b* have co-evolved to this end.

Recent data suggest that mitochondrial metabolic plasticity is essential for malaria parasite development across its complex life cycle with different needs whether in the vertebrate host or in the mosquito vector [34]. During the asexual intraerythrocytic replicative stage, the ATP demands of the parasite are largely met by glycolysis, and the role of the mitochondrial respiratory chain for energy generation seems to be minor [34]. However, bc_1 complex activity remains crucial for the establishment of the transmembrane protonmotive force (which aside from ATP generation is necessary for protein import into the mitochondrion) and for the reoxidation of ubiquinol and the activity of the ubiquinone-dependent dihydroorotate dehydrogenase, and therefore the biosynthesis of pyrimidine [35]. In contrast, the parasite heavily relies on the respiratory chain for energy production during sexual stages and development

within the mosquito. Therefore, an efficient bc_1 complex catalytic activity would be required, with minimal Q_o bypass chemistry and production of damaging SO.

4.4. Atovaquone-resistance mutation Y279S and defective respiratory function

By contrast to PF-Y279, the *Plasmodium*-like modification of the ISP hinge region did not restore - or very weakly- the respiratory growth competence of PF-Y279S, which carried the atovaquone resistance mutation Y279S reported in *P. falciparum* and causing treatment failures. The same behaviour was observed with suppressor mutations in the ISP hinge region as only a very poor compensation of the respiratory growth defect was observed (as shown in Fig.6). This was not unexpected as it was found that substitution of Y279 by a serine, in the yeast native form of the Q_o site, results in a significant decrease of the complex activity [36]. We observed that a tyrosine or a phenylalanine were required at that position to obtain a fully functional enzyme [28].

The severe respiratory defect caused by Y279S -in the *Plasmodium*-like yeast bc_1 complex- is in agreement with the data obtained with *P. falciparum*. In the parasite also, the acquisition of atovaquone resistance is associated with loss of bc_1 complex activity and decreased fitness [37].

5. Conclusions

A *Plasmodium*-like Q_o site is not well accommodated in the yeast bc_1 complex. However, the "inter-species incompatibility" could be overcome by introducing a *Plasmodium*-like hinge region in the ISP, which is likely to restore a correct interaction between the two catalytic partners. The novel chimeric *Plasmodium*-like bc_1 complex seems a reliable model of the parasite enzyme and could be a useful tool to identify new drugs with potential anti-*Apicomplexa* activity targeting the bc_1 complex Q_o site.

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Supplementary data

Strain	Nuclear genome	Cytochrome *b* mutations	Source
wild-type	diploid	wt	
PF-Y279	id	PF-Y279[a]	named PF11 in (Vallières, Fisher, and Meunier 2013)
PF-Y279S	id	PF-Y279S[b]	named PF12 in (Vallières, Fisher, and Meunier 2013)
PF2	id	PF2[c]	(Vallières, Fisher, and Meunier 2013)
PF8	id	PF8[d]	(Vallières, Fisher, and Meunier 2013)
HS1	id	HS1[e]	named HSc in (Vallières, Fisher, and Meunier 2013)
PF-Y279-H53Y	id	[f]	this work
PF-Y279F	id	[g]	this work
JPJ1	*a ade2 his3 ura3 leu2 trp1 rip1::LEU2*		(Beckmanns and Trumpower 1989)
JPJ1 [wt]	id	wt	this work
JPJ1 [PF11]	id	PF-Y279	this work
JPJ1 [PF12]	id	PF-Y279S	this work
CWPF11	W303-1B (mat□ *ade2 his3⁻ leu2 trp1 ura3*)	PF-Y279	this work
CWPF12	id	PF-Y279S	this work

Table S1 Yeast strains.

wt, intronless mitochondrial genome

id, *idem*.

[a] PF-Y279: CCVY$_{133\text{-}136}$VLPW, H141Y, L275F, R283K, MF295-296VL and I299L

[b] PF-Y279S: CCVY$_{133\text{-}136}$VLPW, H141Y, L275F, Y279S, R283K, MF295-296VL and I299L

[c] PF2: CCVY$_{133\text{-}136}$VLPW, H141Y and L275F

[d] PF8: L275F, R283K, MF295-296VL and I299L

[e] HS1: CCVY$_{133\text{-}136}$VLPW, H141F, L275F, F278A and M295L

[f] PF-Y279-H53Y: H53Y, CCVY$_{133\text{-}136}$VLPW, H141Y, L275F, R283K, MF295-296VL and I299L

[g] PF-Y279F: CCVY$_{133\text{-}136}$VLPW, H141Y, L275F, Y279F, R283K, MF295-296VL and I299L

III. Discussion et perspectives

La souche PF-Y279 contenant 10 substitutions dans le site Q_o mimant le site Q_o du parasite a été étudiée. Son complexe bc_1 est sensible à l'atovaquone comme ce qui est observé chez le parasite. L'introduction de la mutation de résistance à l'atovaquone Y279S dans PF-Y279 rend le complexe bc_1 très résistant à l'atovaquone (>250 fois plus résistant que PF-Y279), qui mime bien ce qui se passe chez le parasite.

Mais PF-Y279 et PF-Y279S ont un complexe bc_1 altéré et produisant significativement plus de SO que le contrôle. Un meilleur fonctionnement correct est restauré par des mutations supplémentaires dans le cytochrome *b* : H53Y et Y279F. H53Y a été isolée à partir de la souche PF-Y279 et Y279F a été isolé à partir de la souche PF-Y279S. H53Y est à la surface du cytochrome *b* qui touche la région charnière d'ISP de l'autre monomère. Une phénylalanine à la position 279 reconfirme que Y et F sont interchangeable à cette position (Song, Clain, Iorga, Yi, *et al.* 2015). Des mutations de suppression ont aussi été trouvées dans la région charnière de l'ISP. Enfin, nous avons pu restaurer une meilleure croissance respiratoire de la souche PF-Y279 en replaçant la séquence de cette région charnière de la levure par celle du parasite. Il serait intéressant d'analyser plus à fond ce mécanisme de compensation. Une étude *in silico*, par exemple par dynamique moléculaire, pourrait aider à mieux comprendre au niveau moléculaire comment la modification de la région charnière de l'ISP peut restaurer l'activité enzymatique.

La levure semble un bon outil pour étudier la résistance à l'atovaquone, Les souches « PF » reproduisent bien la sensibilité ou résistance à l'atovaquone observée chez les parasites. Cette même approche pourrait être utilisée pour étudier les autres pathogènes, difficiles à travailler comme *Pneumocystis*, *Toxoplasma*, etc.

Enfin, ces souches de levure « PF » qui miment le site Q_o avec ou sans la mutation de résistance à l'atovaquone pourraient être utilisées pour cribler de nouveaux antipaludiques ciblant le site Q_o et identifier des molécules qui pourraient contourner la résistance à l'atovaquone.

Chapitre I-C. Étude de la sensibilité à l'atovaquone des polymorphismes humains

I. Introduction

Beaucoup de variations génétiques ont été recensées dans le cytochrome *b* humain. Certaines ont été corrélées avec des maladies. Par exemple, D171N (numérotations Humaine) est associée avec la maladie de LHON (Leber hereditary optic neuropathy) et F18L (numérotations Humaine) est retrouvée des cas de glioblastome cérébrale. Ces deux polymorphismes, fréquents dans la population humaine, sont associés à d'autres maladies. Beaucoup d'autres variations sont considérées des polymorphismes ou des mutations silencieuses, sans aucune corrélation avec une maladie.

Des polymorphismes considérés comme totalement silencieux pourraient avoir un effet dans certaines situations physiologiques ou lors de traitement avec des médicaments ciblant de complexe bc_1, comme l'atovaquone et la clomipramine. Il serait intéressant de connaitre les effets de variations du cytochrome *b* sur la sensibilité de l'enzyme à ces molécules. Ceci permettrait d'évaluer les risques du traitement de façon plus personnelle. Les données biochimiques manquent pour évaluer les effets de ces variations. Malheureusement, ces données sont difficiles à obtenir, entre autres, à cause des différences entre les fonds génétiques des lignées cellulaires humaines et de l'impossibilité d'introduire des mutations dirigées dans les gènes mitochondriaux. C'est pourquoi la levure peut être un outil pour analyser l'effet des polymorphismes humains.

Dans cette étude, nous avons analysé une série de mutations répertoriées chez l'Homme. Nous avons testé leurs effets sur l'activité du complexe bc_1 et sa sensibilité à l'atovaquone et à la clomipramine. Cette molécule est utilisée comme antidépresseur mais a aussi une activité anticancéreuse. La clomipramine est un inhibiteur faible du complexe bc_1.

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Human mitochondrial cytochrome *b* variants studied in yeast: not all are silent polymorphisms.

Authors:

Zehua Song[1a], Anaïs Laleve[1a], Cindy Vallières [1b], John E. McGeehan[2], Rhiannon E. Lloyd[3c] & Brigitte Meunier[1c]

[a] equal contribution

[b] Current address: School of Life Sciences, University Park, University of Nottingham, Nottingham, NG7 2RD, UK

[c] Corresponding authors:

Brigitte Meunier, Institute for Integrative Biology of the Cell (I2BC), CEA, CNRS, Université Paris-Sud, Université Paris-Saclay, 91198, Gif-sur-Yvette cedex, France. Tel 00 33 1 69823156

brigitte.meunier@i2bc.paris-saclay.fr

Rhiannon E. Lloyd, Brain Tumour Research Centre, Institute of Biomedical and Biomolecular Sciences, School of Pharmacy and Biomedicine, University of Portsmouth, Portsmouth, UK. Tel 00 44 2392 84 2125

Rhiannon.Lloyd@port.ac.uk

[1] Institute for Integrative Biology of the Cell (I2BC), CEA, CNRS, Université Paris-Sud, Université Paris-Saclay, 91198, Gif-sur-Yvette cedex, France

[2] Molecular Biophysics Laboratories, Institute of Biomedical and Biomolecular Science, School of Biological Sciences, University of Portsmouth, Portsmouth, UK

[3] Brain Tumour Research Centre, Institute of Biomedical and Biomolecular Science, School of Pharmacy and Biomedicine, University of Portsmouth, Portsmouth, UK

Abstract

Variations in mitochondrial DNA (mtDNA) cytochrome *b* (*mt-cyb*) are frequently found within the healthy population, but also occur within a spectrum of mitochondrial and common diseases. *Mt-cyb* encodes the core subunit (MT-CYB) of complex III, a central component of the oxidative phosphorylation system that drives cellular energy production and homeostasis. Despite significant efforts, most *mt-cyb* variations identified are not matched with corresponding biochemical data, so their functional and pathogenic consequences in humans remain elusive. While human mtDNA is recalcitrant

to genetic manipulation, it is possible to introduce human-associated point mutations into yeast mtDNA. Using this system, we reveal direct links between human *mt-cyb* variations in key catalytic domains of MT-CYB and significant changes to complex III activity or drug sensitivity. Strikingly, m.15257G>A (p.Asp171Asn) increased the sensitivity of yeast to the anti-malarial drug atovaquone and m.14798T>C (p.Phe18Leu) enhanced the sensitivity of yeast to the anti-depressant drug clomipramine. We demonstrate that while a small number of *mt-cyb* variations had no functional effect, others have the capacity to alter complex III properties, suggesting they could play a wider role in human health and disease than previously thought. This compendium of new *mt-cyb*-biochemical relationships in yeast provides a resource for future investigations in humans.

Keywords

MT-CYB, mitochondrial DNA, polymorphism, human disease, yeast model, m.14798T>C, m.15257G>A, clomipramine, atovaquone

Introduction

Complex III (or bc_1 complex) of the mitochondrial respiratory chain is central to the cellular energy production process. The complex is anchored within the inner mitochondrial membrane and catalyses the transfer of electrons from ubiquinol to cytochrome *c* and couples this electron transfer to vectorial proton translocation across the inner mitochondrial membrane. The enzyme exists as a functional dimer, consisting of 10 or 11 polypeptides per monomer. All of the subunits are encoded by the nuclear genome, except cytochrome *b* (MT-CYB) (MIM# 516020), which is encoded by mitochondrial DNA (mtDNA). MT-CYB is predominantly a hydrophobic protein consisting of eight transmembrane helices. The subunit contains two haems and forms the two ubiquinol and inhibitor binding sites, called Q_o and Q_i sites (Fig.1A). Complex III is a main site of proton gradient generation, thus of energy-conservation. It is also a main site of reactive oxygen species (ROS) production. ROS are involved in signalling pathways that coordinate both the nucleus and mitochondria to drive beneficial homeostatic responses, but they can also damage cellular components that induce cell death.

In the human population, the *mt*-cyb exhibits a high level of variation. Over 410 missense changes from the *Homo sapiens* mitochondrion complete genome reference sequence (rCRS: GenBank NC_012920.1) have been reported (mitomap.org) and 90 in MSeqDR-LSDB (https://mseqdr.org/MITO/genes/MT-CYB), with some overlap between the various databases.

While a number of these variations are likely to be silent, some variations, especially those located in regions involved in the catalytic activity and inhibitor binding, may induce subtle changes in complex III function. Under exposure to inhibitors, to different energy substrates or to higher energy demands, the altered function of the variant complex III might be revealed, which could affect the fitness of cells and the health of the individuals bearing these variants. Thus what may have previously been considered a silent variation can, under different physiological circumstance, results in a significant effect. Indeed, a recent analysis of 874 genes in 589,306 genomes identified 13 adults harboring highly penetrant mutations that typically cause eight 8 severe Mendelian conditions, with no reported clinical manifestations (Chen *et al.* 2016), which further suggests "silent" "non silent" mutation paradigm is questionable. Several studies have addressed the question of the consequences of *mt-cyb* variations at a population level. For example m.15257G>A, that causes a D to N substitution at position 171 (p.Asp171Asn) of the MT-

CYB polypeptide (Donald R Johns *et al.* 1993) (D R Johns and Neufeld 1993) (Heher and Johns 1993), is associated with LHON and a number of different disease cohorts, whereas m.14798T>C that causes a phenylalanine to leucine amino acid substitution in MT-CYB (p.Phe18Leu), is frequently found in patient-derived glioblastoma biopsy cells (R. E. Lloyd *et al.* 2015) (Larman *et al.* 2012) *(Kirches et al.* 2001), but biochemical data are often lacking. Obtaining such biochemical data is experimentally challenging due to the absence of tools for introducing single variations into mammalian mtDNA. This hiatus in these data could either lead to potentially important mtDNA variations in health and disease being incorrectly dismissed, or to finite resources being erroneously diverted towards the investigation of those which are not important.

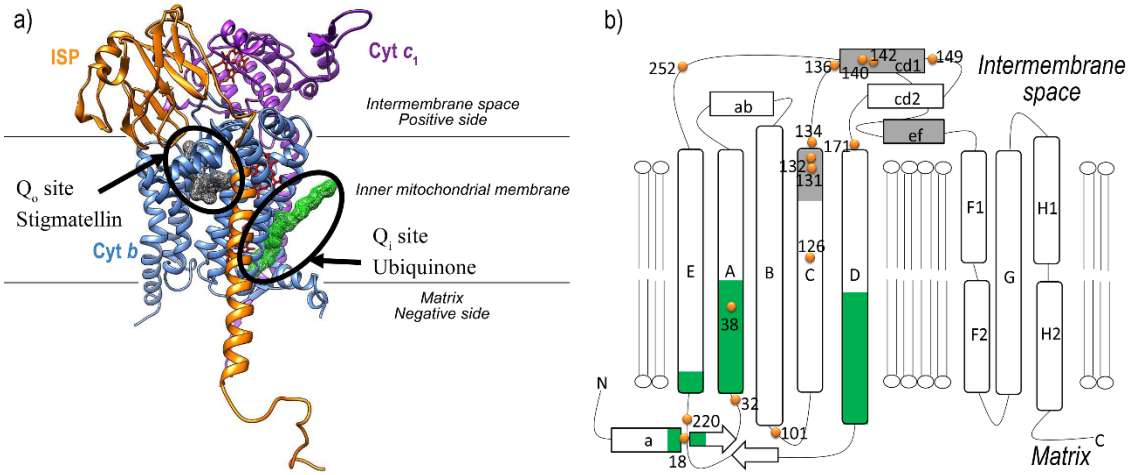

Fig.1 The catalytic core of yeast complex III. (A) Redox active groups are located within three subunits that form the catalytic core: cytochrome c_1 (purple), the iron-sulfur protein/ISP (yellow) and cytochrome *b* (blue), which contains two b-type haems (b_L and b_H) and forms the two quinol binding sites: Q_o (site of quinol oxidation), with bound stigmatellin (grey) and Q_i (site of quinone reduction) with bound ubiquinone (green), located on opposite sides of the membrane. During catalytic turnover, a quinol molecule binds at the Q_o site, is deprotonated, and transfers one electron through the [2Fe-2S] cluster of the ISP and the c-type haem of cytochrome c_1 to cytochrome *c*. Following a bifurcated pathway, a second electron is transferred across the membrane to haems b_L and b_H and delivered to quinone bound at the Q_i site, forming a stable semiquinone. A second quinol oxidation event at the Q_o site completes the Q-cycle with the formation of fully reduced quinol at the Q_i site. In the overall reaction, two molecules of quinol are oxidised to quinone at the Q_o site and one molecule of quinone is reduced to quinol at the Q_i site with the release of four protons to the positive side of the membrane and the uptake of two protons from the matrix. The figure was drawn using the coordinates 2IBZ of yeast complex III. (B) Schematic presentation of cytochrome *b* and location of the residues of interest. The Q_i region is in green; the Q_o region is in grey. The helices are marked by letter (A to H). The positions of the residues (human numbering) studied here are shown by orange dots.

Yeast (*Saccharomyces cerevisiae*) is a convenient model for the study of point mutations in the core subunit of complex III. The organism has well-known advantages. Cells can survive in the absence of respiration relying exclusively on fermentation as an energy source. Therefore the severe malfunction or even the absence of the respiratory enzymes is not lethal. Yeast complex III and especially the catalytic core, is very similar to the mammalian enzyme and several atomic structures are available. Many genetic tools are well developed. Of particular interest, is the mitochondrial transformation technique by which chosen mutations can be introduced into mitochondrially-encoded genes, such as *mt-cyb*. So far, only *S. cerevisiae* and the green alga *Chlamydomonas reinhardtii* are amenable to mitochondrial transformation. Mitochondrial

mutants can then be analysed by a broad range of biochemical/biophysical methods. Using that approach, we have previously assessed the impact of human disease mutations on complex III and used mutants with severe respiratory function defect to explore possible compensation mechanisms (B Meunier *et al.* 2012) (Nicholas Fisher, Bourges, *et al.* 2004) (Nicholas Fisher, Castleden, *et al.* 2004). The yeast model is also an easy-to-use tool for characterizing acquired resistance mutations reported in *mt-cyb* of pathogens after treatment by drugs targeting complex III or to explore the structural basis of the natural differential sensitivity of complex III to inhibitors (Hill *et al.* 2003) (Song, Clain, Iorga, Yi, 2015) (Song, Clain, Iorga, Vallières, *et al.* 2015).

Here, taking advantage of the yeast model, we studied 22 single human variations, including some with reported disease-associations, to obtain biochemical information on their functional impact. We focused on monitoring the effect of mutations that cause non-synonymous amino-acid substitutions located in, or in the vicinity of, the MT-CYB catalytic/binding domains (Fig.1B). In particular, we tested the impact of frequent polymorphisms: 1) p.Phe18Leu located in the Q_i site on complex III sensitivity to the anti-depression compound clomipramine and 2) p.Asp171Asn, the signature of haplogroup J, located nearby the Q_0-site on the complex III sensitivity to the anti-malaria drug atovaquone.

We show that some variants previously reported as "silent" mutations, including some frequent polymorphisms, significantly modify the properties of the yeast complex III, suggesting they may play a greater role in human health and disease than previously thought.

Materials and Methods

Data collection

For each human mutation under investigation, information on the prevalence and heteroplasmy level in normal/healthy and pathologic tissues was obtained from the following five databases: MITOMAP (http://www.mitomap.org/MITOMAP), HmtDB (http://www.hmtdb.uniba.it/hmdb/), mtDB (http://www.mtdb.igp.uu.se/) and MSeqDR-LSDB (https://mseqdr.org/MITO/genes/MT-CYB)) and an in-house glioblastoma (GBM) database (Lloyd *et al.* 2015). Known genotype-phenotype correlations were explored using OMIM (http://www.omim.org/).

Media and chemicals

The following media were used for the growth of yeast: YPD (1% yeast extract, 2% peptone, 3% glucose), YPG (1% yeast extract, 2% peptone, 3% glycerol), YPEth (1% yeast extract, 2% peptone, 3% ethanol), YPGal (1% yeast extract, 2% peptone, 0.1% glucose, 3% galactose). Equine cytochrome *c*, decylubiquinone, atovaquone and clomipramine were purchased from Sigma Aldrich.

Generation of yeast cytochrome b mutants

The plasmid pBM5 carrying the wild type intron-less sequence of *mt-cyb* gene was constructed by blunt end cloning of *mt-cyb* PCR product into the pCRscript vector (Stratagene). The mutagenesis was performed using the Quickchange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's recommendations. After verification of the sequence, the plasmids carrying the mutated genes were used for biolistic transformation. The mitochondrial transformation was performed by microprojectile bombardment as described in (Hill *et al.* 2003),(Brigitte Meunier 2001). The strains are identical except for the mutations introduced in *mt-cyb*, and are all homoplasmic (*i.e.* they contain only one mtDNA population).

Diploids were used in the experiments, except for monitoring the sensitivity of respiratory growth to atovaquone, where the AD1-9 series were used. AD1-9 strain harbours multiple deletions in the ABC transporter genes that render the strain more sensitive to drugs than standard yeast strains (α ura3 his1, yor1Δ ::hisG, snq2Δ::hisG, pdr5Δ:: hisG, pdr10Δ ::hisG, pdr11Δ ::hisG, ycf1Δ ::hisG, pdr3Δ ::hisG, pdr15Δ ::hisG, pdr1Δ ::hisG) (kindly given by M. Ghislain, UCL, Belgium). We previously found that while the yeast complex III is highly sensitive to atovaquone, the drug cannot reach the complex unless ABC transporters are deactivated (Hill *et al.* 2003).

Preparation of mitochondria and measurement of cytochrome c reductase, activity

Bovine mitochondrial samples were kindly given by Prof. Peter Rich (UCL, UK). Yeast mitochondria were prepared as in (Lemaire and Dujardin 2008). Briefly, yeast grown in YPGal medium were harvested at mid-log phase. Protoplasts were obtained by enzymatic digestion of the cell wall using zymolyase in an osmotic protection buffer. Mitochondria were then prepared by differential centrifugation following osmotic shock of the protoplasts. Mitochondrial samples were aliquoted and stored at -80 °C. Concentration of complex III in the mitochondrial samples was determined from dithionite-reduced optical spectra, using ε=28.5 mM^{-1} cm^{-1} at 562 nm *minus* 575 nm. Decylubiquinol-cytochrome *c* reductase activities were determined at room temperature by measuring the reduction of cytochrome *c* (final concentration of 20 μM) at 550 nm *versus* 540 nm over 1-min time-course in 10 mM potassium phosphate pH 7, 0.01% (w/v) lauryl-maltoside and 1 mM KCN. Mitochondria were added to obtain a final concentration of 5-30 nM of complex III. Activity was initiated by the addition of decylubiquinol, a synthetic analogue of ubiquinol (final concentration of 40 μM). Initial rates were measured as a function of the decylubiquinol concentration. Each measurement was repeated three times and the values obtained were averaged. Activity (turnover) were estimated as cytochrome *c* reduction rates per complex III.

Inhibitor titration

Cytochrome *c* reduction activity was measured as described above in the presence of increasing concentrations of inhibitors (six to ten different concentrations). Each measurement was repeated two or three times and averaged. The mid-point inhibition concentrations (IC_{50}) were determined from the titrations and the IC_{50} values were normalized by the concentration of complex III.

Respiratory growth assays

Yeast strains were grown in 5 ml of YPEth medium with increasing concentration of drug. Cultures were inoculated from one day-old cultures on YPG to an OD_{600}nm of 0.2 and incubated at 28°C with vigorous shaking to maintain good aeration. Cell densities measured as OD_{600}nm were estimated two or three days after inoculation.

Results and discussion

Spectrum of human mt-cyb variations analysed in yeast

The 22 human polymorphisms chosen for analysis in yeast are presented in Table 1. With the exception of one variation (m.15048G>C), reported in a single GBM-patient (R. E. Lloyd *et al.* 2015), all variations were reported as polymorphisms in one or more of the mitochondrial databases. Eleven polymorphisms have no known disease-association: m.15122A>G, 15147C>T, 15171G>A, 15191T>A, 15258GA>AG,

15048G>C, 15404G>C, 15138A>G, 15152G>A, 15153G>A and 15502C>G, and were rare, being found in typically 10 cases or less in the normal/healthy human population. Nine polymorphisms had one or more reported disease-associations: m.15140G>A, 15164T>C, 15257G>A, 15258A>G, 14798T>C, 14841A>G, 14858G>A, 15047G>A and 15500G>A. The top three most frequently recorded polymorphisms in normal healthy cases, were also the most frequently recorded in disease-associated cases (in descending order of patient frequency): m.14798T>C, 15257G>A and 15047G>A. The individual *mt-cyb* variations were associated with one or more of the following type of disorders: cardiological (e.g. cardiomyopathy and Noonan syndrome); neurological (e.g. LHON, Parkinson's and Schizophrenia); metabolic (e.g. diabetes); and cancers (e.g. breast, thyroid, GBM, pituitary adenoma, oral squamous cell carcinoma and lung), there were no genotype-phenotype correlations in the OMIM database, with the exception of m.15257G>A, which is associated 9% of LHON cases (D R Johns and Neufeld 1993) (Heher and Johns 1993) (Donald R Johns *et al.* 1993).

The polymorphisms were chosen on the basis of the location of the residues in the structure of complex III. Nine variations (at positions 126, 132, 134, 140, 142, 149 and 171) are located in or nearby the Q_o site. Eight variations (at positions 18, 32, 38, 101 and 220) are in or nearby the Q_i site. Five variations (at positions 131, 136 and 252) are located in possible proton routes from the Q_o site (Table 1 and Fig.1B).

Human						Yeast
Nucleotide substitution*	amino acid substitution	Normal, healthy (number of cases)	Pathologic (disease) (number of cases)	% Het (disease)	Predicted effect on complex III (based on yeast model and on structure)	Matching amino acid substitution [c]
Qo domain : atovaquone sensitivity						
m.15122A>G	T126A	5	0	n/a	none	T127I
m.15140G>A	V132I	1	3(breast and thyroid cancer)		slight modification of ato sensitivity	CCV 133-135 VLP
m.15147C>T	P134L	n	0	n/a	slight modification of ato sensitivity	
m.15164T>C	F140L	6	1(diabetes)		slight modification of ato sensitivity	H141Y/F
m.15171G>A	G142E	1	0	n/a	severely decreased activity, decreased ato sensitivity	G143A
m.15191T>A	L149M	2	0	n/a	decreased ato sensitivity & activity	L150F
	D171 (rCRS)					S172D
m.15257G>A	D171N	319 (haplogroup J)	51 [a]	100 (GBM)	increased sensitivity to ato	S172N
m.15258A>G	D171G	24	2 (diabetes type2/LHON)		increased sensitivity to ato	S172G
m.15258GA>AG	D171S	n	0	n/a	increased sensitivity to ato	S172 (wt)
Q_i domain : clomipramine sensitivity						
	F18 (rCRS)					I17F
m.14798T>C	F18L	4410	233 [b]	99 (GBM)	increased sensitivity to clom	I17 (wt)

m.14841A >G	N32S	1	1 (LHON)		increased sensitivity to clom, decreased activity	N31S
m.14858G >A	G38S	10	2 (mental disorder, diabetes)		increased sensitivity to clom	G37S
m.15048G >C	G101A	n	0	n/a	none	
m.15047G >A	G101S	50	3 (Thyroid cancer, diabetes type II)		none	
m.15048G >A	G101D	0	1 (GBM)	17 (GBM)	decreased sensitivity to clom	G100D
m.15404T >C and 15406C>A	F220L	2	0	n/a	increased sensitivity to clom	F225L
Qo domain : proton pathway						
m.15138A >G	Y131C	n	0	n/a	none or slightly decreased activity	Y132F
m.15152G >A	G136S	3	0	n/a	severely decreased activity	G137R
m.15153G >A	G136D	4	0	n/a	severely decreased activity	G137E
	D252 (rCRS)					H253D
m.15502C >G	D252E	1	0	n/a	none	H253E
m.15500G >A	D252N	1	2 (diabetes/GBM)	5 (GBM)	none	H253N

Table 1. Prevalence of human *mt-cyb* variations in the normal and pathologic population and their predicted effect on the complex III properties.

n: undetermined number of cases. n/a: not applicable.

*: Nucleotide variations are relative to the *Homo sapiens* mitochondrion, complete genome (GenBank NC_012920.1)

[a] GBM, pituitary adenoma, Parkinson's, CADASIL, Noonan syndrome, Schizophrenia, LHON, oral squamous cell carcinoma, lung cancer

[b] GBM, thyroid tumours, LHON, prostate cancer, type I endometrial carcinoma, schizophrenia, breast cancer, cardiomyopathy, optic atrophy, CPEO, pituitary adenoma, oral cavity carcinoma, Warthin tumour, OXPHOS system deficiency, Noonan syndrome

[c] Mutations were introduced in yeast cytochrome *b* using the mitochondrial transformation technique (Materials and Methods) with the exception of G137E that had been obtained by random mutagenesis.

Mutations within or nearby the Q_o site influence atovaquone sensitivity

Atovaquone (used in combination with proguanil, and marketed as Malarone®) is a popular prophylactic drug that also shows high efficiency in the treatment of uncomplicated *Plasmodium falciparum* malaria. The compound is also used to treat *Pneumocystis jirovecii* pneumonia, *Toxoplasma gondii* toxoplasmosis, and other infections. Atovaquone inhibits complex III by binding in the Q_o site, as revealed by the resolution of

the atomic structure of yeast complex III with bound atovaquone (PDB code 4PD4) (Birth, Kao, and Hunte 2014) and by the Q_o site location of point mutations found in the *mt-cyb* of atovaquone-resistant parasites (Hill *et al.* 2003) (Mather *et al.* 2005).

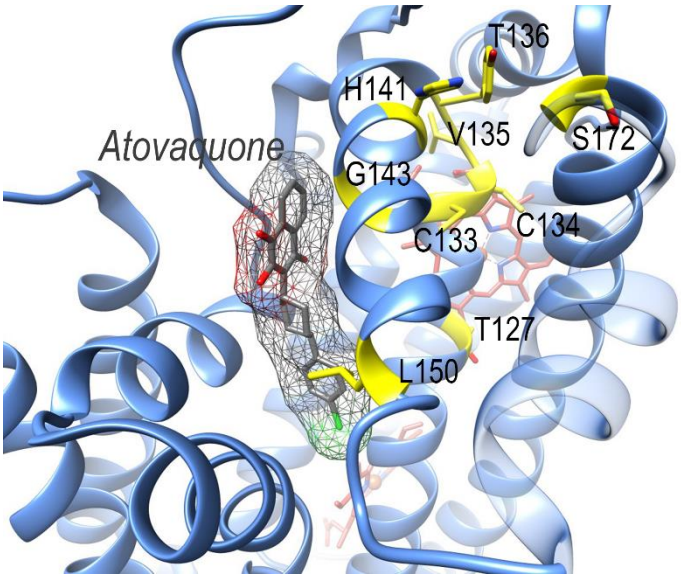

Fig.2 Location of the residues in yeast Q_o domain with bound atovaquone. Atovaquone (green) is bound is a position similar to stigmatellin (Fig. 1A). Haem b_L is in red. Yeast numbering is used. Residues p.Gly143, p.Leu150, p.Thr127, p.Cys133, p.Cys134, p.Val135, p.Tyr136, p.His141 and p.Ser172 are coloured in yellow and their sidechains are shown. The figure was drawn using the coordinates 4PD4 of yeast complex III.

The complex III of the malaria parasite - and yeast - is highly sensitive to atovaquone while the mammalian complex III is naturally more resistant to atovaquone, which is expected of an anti-malarial compound used to treat humans. The IC_{50} (mid-point inhibition concentration) values per nM complex III were estimated to be 75 and 4 nM for the bovine and the yeast enzymes, respectively (Vallières, Fisher, and Meunier 2013). The IC_{50} value for the malaria parasite enzyme was reported to be similar to the yeast value, around 3 nM (Biagini *et al.* 2008). The differential sensitivity between mammalian and *Plasmodium* (and yeast) is likely to be due to amino-acid variations in the Q_o site, more specifically, variations of MT_CYB residues 275, 278 and 295, as suggested by mutational analysis using the yeast model (Vallières, Fisher, and Meunier 2013).

Nine of the chosen human polymorphisms (Table 1) are located at seven positions in or near the atovaquone binding pocket, and thus might modify the sensitivity of the enzyme to the drug (Fig.2). In order to explore this, we generated yeast mutants harbouring equivalent MT-CYB mutations and analysed their atovaquone sensitivity; we also reviewed previously published data.

The human p.Gly142Glu (yeast G143) and p.Leu149Met (yeast L150) (Table 1) are located in the atovaquone binding site (Fig.2). We predict that these substitutions would alter the sensitivity to atovaquone but also interfere with catalytic activity, by hindering the correct binding of the substrate ubiquinol. In yeast, mutation p.Gly143Ala– a minor addition of a single carbon atom compared to the addition of a bulky acidic group in G143E - confers a 9-fold increase in atovaquone resistance (Fisher and Meunier 2005, Supp. Table S1). G143D was studied in a bacterial complex III and was shown to decrease enzymatic activity severely (Daldal *et al.* 1989, Supp. Table S1). It is likely that in human complex III, p.Gly142Glu would also result in an inactive enzyme. Mutation p.Leu149Met would have a less dramatic effect on the enzyme activity. In yeast, mutation p.Leu150Phe results in a 3-fold increase in atovaquone resistance with the loss of 50 % of complex III activity (Hill *et al.* 2003, Supp. Table S1).

Human variants p.Val132Ile, p.Pro134Leu and p.Phe140Leu (fig 1) are located in the vicinity of the Q_o pocket (residues 133, 135, 141 in yeast (Fig.2)). We have previously observed that, in yeast, atovaquone sensitivity was modified by mutations in the region 133 to 141 (Supp. Table S1). The residues are not in direct contact with bound atovaquone in the crystal structure but mutations could indirectly modify the binding pocket and, by consequence, slightly alter the binding of the drug (Vallières, Fisher, and Meunier 2013) (Nicholas Fisher and Meunier 2005). p.Thr126Ala is located distal from the Q_o binding pocket. As expected, the matching yeast mutation p.Thr127Ile had no effect on complex III activity and atovaquone sensitivity (Hill *et al.* 2003, Supp. Table S1).

We then sought to determine whether mutations of human residue 171 (172 in yeast) (Table 1), also located in the vicinity of the Q_o site (Fig.2), could modify the sensitivity to atovaquone.

The yeast residue, S172 (wt), which also reflects a rare polymorphism in humans, was replaced by D, the residue present in the human reference sequence; N, the frequent polymorphism and marker of haplogroup J in humans, and finally; G, also a polymorphism in humans. Modified IC_{50} values to atovaquone were observed (Fig. 3A). Complex III with p.Ser172Asp was found to be more resistant (IC_{50} of 11.5) than the wt, G and N (IC_{50}s of 7, 7.5 and 5, respectively) to atovaquone. None of the mutations had a major effect on the catalytic activity of the complex (Supp. Table S1). We then tested whether these small changes in sensitivity could be observed when monitoring the respiratory growth of mutants and control strains. As shown in Fig.3B, p.Ser172Asn, p.Ser172Gly and the wt displayed an increased sensitivity compared to p.Ser172Asp (and to p.Leu150Phe, mentioned above).

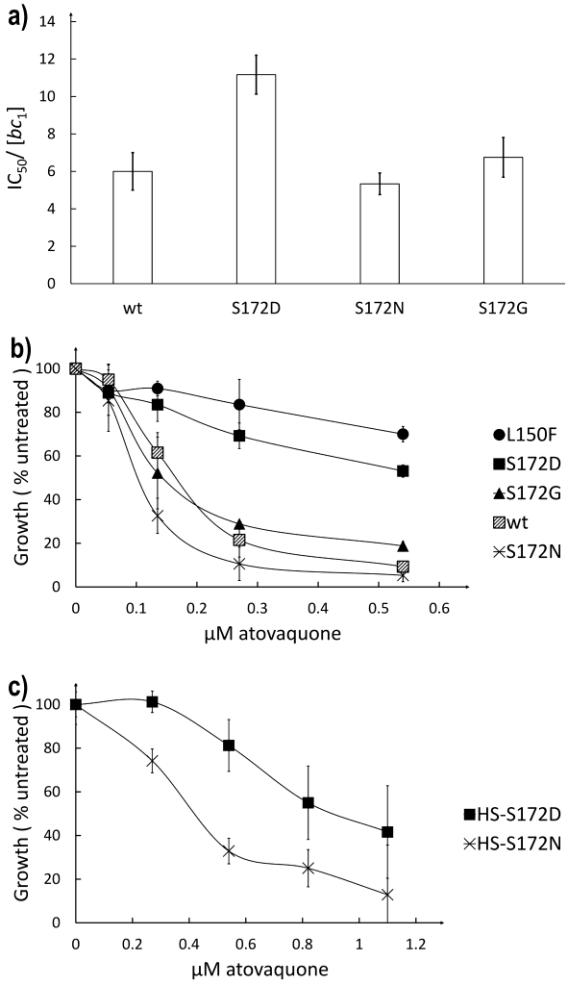

Fig.3 (A) Atovaquone sensitivity of yeast mutant and wild type control complex III. IC_{50}s, mid-point inhibition concentrations, were estimated from the titration measurements and reported per complex III (Materials and Methods). Each measurement was repeated three times and averaged. Error bars represent standard deviations. (B & C) Sensitivity of yeast mutant and wild type control respiratory growth to atovaquone. Atovaquone sensitivity is presented for each strain as the percentage of growth relative to wild type control, *i.e.* untreated. Each measurement was repeated at least twice and averaged. Error bars represent standard deviations. **(B) Respiratory growths of strains harbouring single mutation p.Ser172Asp,N,G and p.Leu150Phe. (C) Respiratory growths of strains with multiple changes (HS) in the Q_o site that replace yeast residues by human equivalents.**

In order to confirm the differential sensitivity induced by the polymorphisms at position 172, we tested whether the same behaviour could be observed in mutant strains that harbour a complex III Q_o site that more closely resembled that of the human enzyme. To this end, two strains were genetically constructed. In addition to the amino acid substitution at position 172, eight mutations in the Q_o site were introduced by replacing the yeast residues by their human equivalents: $CCVY_{133\text{-}136}$VLPW, p.His141Phe, L275F, F278A and M295L. The resulting strains, called HS-p.Ser172Asp and HS-p.Ser172Asn, were tested for the sensitivity to atovaquone (Fig. 3C). Both strains were inhibited at slightly higher doses of atovaquone than the control strain. This was expected as we have previously observed that yeast to human amino acid substitutions at positions 275, 278 and 295 resulted in an increased resistance to the drug (Vallières, Fisher, and Meunier 2013). Despite this, HS-p.Ser172Asn was still more sensitive to atovaquone than HS-p.Ser172Asp.

Mutations within and near by the Q_i site modify clomipramine sensitivity

The main clinical use of clomipramine is as a tricyclic antidepressant but it is also showing promise in pre-clinical studies for the treatment of glioma, potentially via a mitochondrial targeted mechanism. In addition to its main mode of action, which is to bind non-selectively to 5-hydroxytryptaimine and noradrenaline uptake receptors, which endows it with antidepressant activity (Rang, Dale, and Ritter 2011), the drug has been shown to induce apoptosis in malignant glioma via the direct release of cytochrome *c* which stimulates the p-c-Jun pathway and triggers the caspase cascade (Levkovitz *et al.* 2005) (Pilkington, Parker, and Murray 2008). Furthermore, clomipramine has been shown to impair cell respiration and inhibit complex III in glioma, thus implicating the complex III in the glioma cell killing effect of this drug (Higgins and Pilkington 2010)

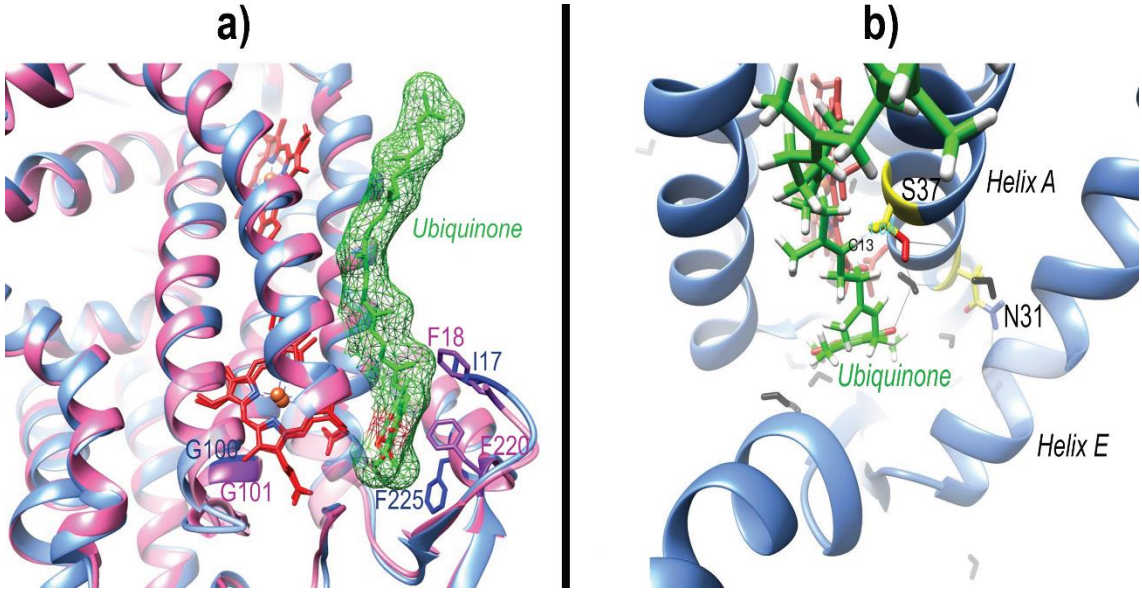

Fig.4 (A) Comparison of the yeast and mammalian Q_i sites. Superposition of yeast (blue) and bovine (pink) cytochrome *b* structures. Ubiquinol is in green and haem b_H, in red. The figure was drawn using the coordinates 4PD4 and 1PP9 of yeast and bovine complex III, respectively. The two structures were superposed using Chimera. Residues p.Ile17, p.Gly100 and p.Phe225 of yeast cytochrome *b* and p.Phe220, p.Gly101 and p.Phe220 of mammalian cytochrome *b* are shown. **(B) Possible interactions between p.Asn31 and helix E, and between p.Gly37Ser and ubiquinol.** Cytochrome *b* is colored in blue, ubiquinol in green, p.Asn31 and p.Ser37 in yellow, waters as black sticks and haem b_H in red. Possible hydrogen bonds are indicated in black lines. Minimum distance between p.Ser37 and ubiquinol p.Cys13 hydrogen is 1.0 Å. The structure was drawn using 2IBZ. *In silico* mutation p.Gly37Ser was introduced using Chimera.

We tested clomipramine inhibitory effect on the catalytic activity of bovine and yeast complex III, using purified mitochondria, and determined the IC_{50}s. The drug was found to be a weak inhibitor of the enzymes, with IC_{50} values of 24± 5 and 3.4± 0.1 □ M per nM of bovine and yeast complex, respectively. Bovine complex III seems thus naturally less sensitive to clomipramine than the yeast enzyme. Complex III possesses two distinct inhibitor binding sites, the Q_o (that binds atovaquone) and the Q_i site. Comparisons of the bovine and yeast Q_i sites reveal structural differences that could be responsible for the observed differential sensitivity to clomipramine, in particular the replacement of I17 present in yeast by F present in the mammalian enzyme (Fig. 4A). Intriguingly, we have previously identified a parallel amino acid substitution (p.Phe18Leu) in 30% of biopsy derived GBM samples, and predicted it to affect complex III inhibitor binding (R. E. Lloyd *et al.* 2015).

We thus sought to determine whether variations in the Q_i site could influence sensitivity of complex III to clomipramine.

A shown in Fig. 5, the mutation p.Ile17Phe (replacing the yeast amino-acid by the human residue) resulted in a two-fold decrease in clomipramine sensitivity. Thus the phenylalanine naturally present at that position (F18) in mammalian MT-CYB could explain, at least in part, the decreased clomipramine sensitivity of the mammalian enzyme compared to the yeast enzyme. The frequent polymorphism p.Phe18Leu in humans – corresponding to yeast wild type I17 is likely to result in an increase sensitivity to the drug.

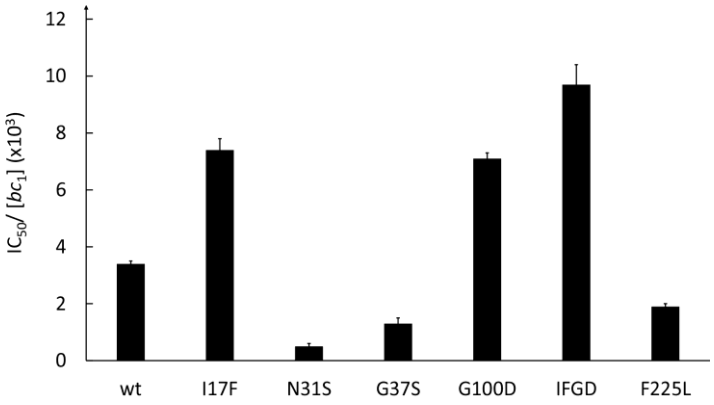

Fig.5 Clomipramine sensitivity of yeast mutant and wild type control complex III. IC_{50}s, mid-point inhibition concentrations, were estimated from the titration measurements and normalized for complex III concentration (Materials and Methods). Each measurements was repeated three times and averaged. Error bars represent standard deviations. IFGD: p.Ile17Phe+p.Gly100Asp.

Other mutations in or in the vicinity of the Q_i site were also investigated: p.Asn31Serp.Asn31Ser (p.Asn32Ser and rare polymorphism in humans,), p.Gly37Ser (p.Gly38Ser and a rare polymorphism in humans) (Fig. 4B), p.Gly100Asp (p.Gly101Asp reported in a GBM-patient; Lloyd *et al.* 2015) and p.Phe225Leu (structurally similar to p.Phe220Leu in humans, rare polymorphism). p.Gly100Asp resulted in decreased clomipramine sensitivity, whereas p.Asn31Serp.Asn31Ser, p.Gly37Ser and p.Phe225Leu increased the sensitivity to the drug (Fig.5). Interestingly, the double mutation p.Ile17Phe-p.Gly100Asp, had an additive effect, further decreasing clomipramine sensitivity. These data substantiate the hypothesis that the Q_i site is a clomipramine target and Q_i site mutations can affect clomipramine sensitivity. None of these mutations had a major impact on complex III activity, except p.Asn31Serp.Asn31Ser that resulted in a two-fold decrease: its activity, was 25 $\pm$ 0.8 s^{-1} compared to 56 $\pm$ 4.6 s^{-1} for the wild type (Supp. Table S1).

Examination of the structure of the Q_i pocket domain (Fig.4A) showed that replacement of the small residue I17 by a bulky phenylalanine (p.Ile17Phe) could sterically hinder the binding of clomipramine, which is a far larger molecule than the natural ligand, whereas the substitution of F225 by the smaller leucine (p.Phe225Leu) would leave more space for the binding of the drug.

The effect of p.Gly100Asp is less straightforward to explain as the residue is not located directly in Q_i site. However, the replacement of the small glycine by the bigger and charged aspartate might indirectly alter the pocket reducing its affinity for the drug. That major change (p.Gly100Asp) had minimal effect on the catalytic activity of the complex III. As the enzyme seems to accommodate p.Gly100Asp, it is likely that the less drastic substitutions reported in humans, p.Gly101Ala and p.Gly101Ser would also have minimal impact on the enzyme properties.

The increased clomipramine sensitivity induced by p.Asn31Serp.Asn31Ser and p.Gly37Ser might be caused by modifications of the binding pocket (Fig.4B). As we have previously reported, the equivalent residue to N31 in human (N32) participates in stabilizing hydrogen bonds with key residues in the Q_i site (Rhiannon E. Lloyd and McGeehan 2013). Introduction of a serine might slightly modify the local structure which would stabilize clomipramine but decrease the catalytic activity. A serine at position 37 might slightly tighten the drug binding without impairing the activity.

Q_o proton pathway mutations influence complex III activity

As explained in Fig.1, during the catalytic reaction, a quinol is oxidised in the Q_o site. Upon oxidation, two protons are released in the solvent phase at the intermembrane side of the inner membrane. Several residues and water molecules are likely to form pathways for proton movement (Hunte, Palsdottir,

and Trumpower 2003a) (Palsdottir *et al.* 2003). The conserved residue E272 seems to be one of the main actors in the proton relay (Wenz *et al.* 2006) (Seddiki *et al.* 2008) (Crofts *et al.* 2013). H253 and Y132 also interact with water molecules and might be involved in forming proton routes. G137, in contact with the conserved S140, is located in the vicinity of this water-polar residue network (Supp. Fig.S1). We hypothesise that mutation of these residues could impair the proton movement and by consequence the catalytic activity of complex III.

In order to test this hypothesis systematically, we replaced H253 in yeast by D (human rCRS), E (reported as a polymorphism) and N (reported polymorphism and in GBM) (Table 1). H, D, E and N are polar residues. In parallel, we tested the effect of introducing the apolar residue F at that position, even though that mutation has not been observed in humans. We also assessed the effect of replacing the polar residue Y132 by the apolar F. Two other mutations were tested p.Gly137Glup.Gly137Glu and p.Gly137Arg, corresponding to the human polymorphisms p.Gly136Asp and p.Gly136Ser. We measured the complex III activity and monitored the respiratory growth of the yeast mutants (Fig. 6).

Replacement of H253 by F decreased the electron transfer activity of the complex by 40% (Fig. 6A). Introduction of the human residues D, E or N resulted in a complex with a higher electron transfer activity (130-160% of the control activity). The introduction of an apolar residue at position 253 probably slows down proton movement and concomitant electron transfer. D, E or N might facilitate the proton translocation and thus increase the electron transfer rate. In organisms with a non-protonable residue at position 272, a glutamate at position equivalent to H253 was observed, suggesting a role in proton movement (Brasseur *et al.* 2002).

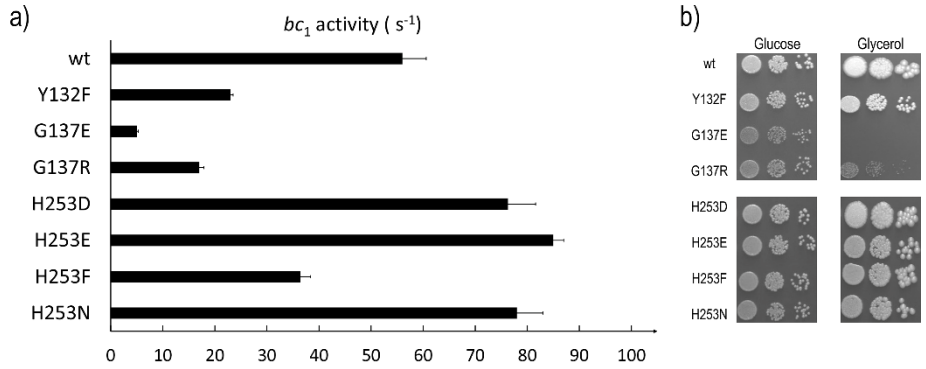

Fig.6 (A) Complex III activity in wild type control and mutants. The ubiquinol cytochrome *c* reductase assays were performed as described in Materials and Methods. The activities measured at saturated substrate concentrations (40 mM decylubiquinol) were reported relative to complex III concentration. The measurements were repeated at least twice and averaged. The error bars represent standard deviations. **(B) Respiratory growth of wild type control and mutants.** Serial dilutions in water of cells pre-grown on glucose plates were spotted on plates containing either glucose (YPD, fermentative medium) or glycerol (YPG, respiratory medium) and incubated for two (YPD) to four days (YPG) at 28 °C.

The mutations of H253 had no effect on the respiratory growth competence (Fig.6B). The substitution p.Tyr132Phe had a more severe impact and decreased complex III activity by around 50% (Fig.6A) resulting in a lower respiratory growth competence (Fig.6B). The apolar residue F is likely to disrupt the water network and proton routes. Examination of the structure suggests that a polar residue is required at that position. Thus the replacement of the tyrosine by a cysteine (human polymorphism p.Tyr131Cys) might have only a mild effect.

In contrast to p.Tyr131Cys, the human polymorphisms p.Gly136Ser and particularly p.Gly136Asp (Table 1) are expected to have dramatic effects. In yeast, the equivalent mutations p.Gly137Arg and p.Gly137Glup.Gly137Glu caused, respectively, 70% and 90% decrease in III activity and a very severe respiratory growth defect (Fig.6). The introduction of charged or polar residues at that position could severely impair the transfer of protons from the Q_o site towards the negative side of the membrane, as previously suggested (Tron and Lemesle-Meunier 1990)(Bruel *et al.* 1995).

Clinical relevance and future prospects

The continual advancement of sequencing technologies has allowed the identification of increasing numbers of *mt-cyb* variations in humans. However, it remains impossible to introduce single mutations into human mtDNA and thus observe any direct genotype-phenotype correlations at the molecular or cellular level. Although the technology exists to produce custom cybrids, such cell lines are likely to contain several mtDNA variations which could contribute to phenotype (Singh *et al.* 2014). Also, the process of creating cybrids can introduce irreversible epigenetic changes (Smiraglia *et al.*, 2008), as well as nuclear genome instability and mutation (Singh *et al.*, 2005), further confounding isolating a single mtDNA mutation as contributing factor. Also, under normal circumstances, subtle changes in complex III properties might be missed, and it is only when cells are forced to use alternative metabolic pathways through changes in their cellular environment, or in the presence of inhibitors, as we have shown here, that they are revealed.

Our focus was to use the yeast model system, whose mtDNA is amenable to genetic manipulation, to investigate the direct functional consequences of human MT-CYB mutations on corresponding complex III properties. Our results reveal that most of mutations that cause amino acid replacements in key catalytic regions of MT-CYB, affected the sensitivity of complex III to drugs and/or complex III activity. Although the MT-CYB mutations investigated here could exert a more severe effect in yeast cells than in human cells where they might be present at lower heteroplasmy, the potential clinical relevance of the mutations to humans and recommendations for future work are discussed below.

In this study, we showed seven amino acid replacements increased the sensitivity of yeast complex III to either atovaquone or clomipramine (mutations corresponding to the humans polymorphisms p.Asp171Asn, p.Asp171Gly, p.Asp171Ser, p.Phe18Leu, p.Asn32Ser, p.Gly38Ser and p.Phe220Leu). This is particularly significant finding for p.Asp171Asn and p.Phe18Leu, as they frequently occur in the normal/healthy individuals, as well as those with disease. p.Asp171Asn- and p.Phe18Leu-carriers (along with carriers of the other rarer polymorphisms) might respond more strongly to atovaquone and clomipramine, respectively: their complex III would be inhibited at lower doses than the enzyme of controls, which could lead to respiratory chain defect and impaired metabolism, especially in cells that heavily rely on the respiratory chain for their energy supply. Thus the polymorphisms would be disadvantageous for individuals/patients who are prescribed the drugs for the treatment of malaria/depression, due to the potential side-effects. Such side effects are likely to be even more marked in individuals that already harbor mitochondrial/metabolic disorders. The converse would be expected for individuals carrying the rarer polymorphisms that decrease atovaquone/clomipramine sensitivity. As new Q_o and Q_i site inhibitors are being developed as anti-malarial agents (J Stone Doggett *et al.* 2012) (A. Nilsen *et al.* 2013) (Allison M. Stickles, Justino de Almeida, *et al.* 2015), it would seem pertinent to determine their selectivity and efficacy on p.Asp171Asn or p.Phe18Leu-carrying human cells.

While enhanced sensitivity of complex III to atovaquone or clomipramine may be a disadvantage for individuals carrying the polymorphism p.Asp171Asn or p.Phe18Leu, it might be an advantageous phenomenon for the selective killing of cancer cells, e.g. glioma, in which p.Asp171Asn and p.Phe18Leu are reported. Further investigation into the role of mitochondrial genetic background in governing the potential selectivity/efficacy of chemotherapeutic strategies that target complex III on human cancer cells is therefore warranted. For example, the presence of p.Asp171Asn or p.Phe18Leu could be used as an inclusion criterion for pre-clinical trials testing either atovaquone or clomipramine on human cancer cells or in rodent models. Retrospective analyses could then be conducted to determine whether p.Asp171Asn/p.Phe18Leu carriers responded better to treatment than those that did not carry the mutation.

Also in this study, we identified three amino acid replacements that severely decreased complex III activity, namely p.Gly136Ser, p.Gly136Asp and p.Gly142Glu. These mutations, due to their impact on yeast or bacterial complex III, are expected to be severely pathological in humans. This could explain why they are rarely detected in the human population. The few normal, healthy individuals that harbour these mutations could be examples of so called 'resilient' individuals that despite possessing mutations for severe Mendelian conditions, have no reported clinical manifestations of the indicated disease (Chen *et al.* 2016). Samples from these patients could be of interest to scientists who are seeking to discover genetic and environmental modulators that buffer the effects of, in this case, potentially deleterious mitochondrial mutations.

Other mutations (p.Asn32Ser and p.Leu149Met) would result in a milder effect on complex III activity, which could still impact the overall activity of the respiratory chain. These mutations might also be pathogenic, as previously predicted for p.Asn32Ser, a LHON-associated mutation (Lloyd & McGeehan 2013).

Finally, several substitutions are not expected to impair complex III activity and are unlikely to be pathogenic: p.Phe18Leu, p.Gly38Ser, p.Gly101Ala, p.Gly101Ser, p.Gly101Asp, p.Thr126Ala, p.Asp171Asn, p.Asp171Gly, p.Asp171Ser, p.Phe220Leu, p.Asp252Glu and p.Asp252Asn. This is interesting because the frequently occurring polymorphisms p.Asp171Asn and p.Phe18Leu have been proposed to have a pathogenic role in numerous diseases. Through association, p.Asp171Asn has been proposed to cause one of the mildest LHON phenotypes, where patients have difficulty in perceiving hand motion (Donald R Johns *et al.* 1993). However, results presented here from yeast cells suggest that p.Asp171Asn is unlikely to contribute to LHON in isolation and possibly other co-occurring mutations, either alone or in combination with p.Asp171Asn, are more likely to be responsible, as suggested previously (Mackey *et al.* 1996). Similarly, through association p.Phe18Leu (and the rarer p.Gly101Asp and p.Asp252Asn) have been proposed to contribute to several diseases, including GBM (Lloyd *et al.* 2015). Results presented here in yeast cells suggest p.Phe18Leu and the other mutations are unlikely to be responsible, but could play a role in clomipramine sensitivity.

We show that several human-associated mutations, when introduced into yeast mtDNA, affect complex III activity and/or drug sensitivity, with no clear relationship between the two parameters. These finding suggests, while complex III mutations could play a greater role in human health and disease than previously thought, they also highlight once again the challenges associated with predicting the functional importance of amino-acid substitutions in MT-CYB, presumably due to the complicated cross-talk that exists between mitochondrial function and the cellular environment. However, the yeast model system provides the opportunity to probe such relations in an uncomplicated genetic background under different physiological conditions, including in the presence of inhibitors. This compendium of new *mt-cyb-*

biochemical relationships in yeast presented here will help clinicians and scientists decide which variations to prioritise for future pre-clinical investigations in humans.

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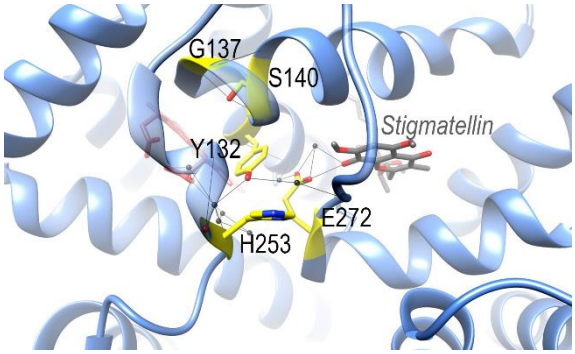
Supplementary data



Supp. Figure S1. Location of residues in possible proton routes in the yeast Q_o pocket. Yeast cytochrome *b* is colored in blue, stigmatellin in grey, haem b_L in red, water molecules in black. Residues p.Tyr132, p.Gly137, p.Ser140, p.His253 and p.Glu272 are colored in yellow and the sidechains are shown. Hydrogen bonds are calculated and generated by Chimera, shown by black lines. The figure was drawn using the coordinates 3CX5 of yeast complex III.

Supp. Table S1. Yeast MT-CYB mutants: complex III activity and sensitivity to inhibitors

Q_o domain : atovaquone sensitivity			
	complex III (% control)	**atovaquone sensitivity (IC_{50} mutant/IC_{50} control)**	**references**
p.Thr127Ile	81	1	(Hill *et al.* 2003)
CysCysVal $_{133-135}$ ValLeuPro	100	3.5	(Fisher and Meunier 2005)
p.His141Tyr	52	0.5	(Vallières *et al.* 2013)
p.His141Phe	68	0.4	(Fisher and Meunier 2005)
p.Gly143Ala	87	9	(Fisher and Meunier 2005)
p.Gly143Asp [a]	inactive	nd	(Daldal *et al.* 1989)
p.Leu150Phe	54	3	(Hill *et al.* 2003)
p.Ser172Asp	72.3 ± 5.5	1.9	This work
p.Ser172Asn	86.5 ± 9.2	0.9	This work
p.Ser172Gly	110 ± 25.4	1.1	This work
Q_i domain : clomipramine sensibility			
	***bc*$_1$ complex activity (s^{-1})**	**clomipramine sensitivity (IC_{50} mutant/IC_{50} control)**	
p.Ile17 (WT)	56 ± 4.6	1	This work
p.Ile17Phe	46 ± 15.1	2.2	This work
p.Asn31Ser	25 ± 0.8	0.15	This work
p.Gly37Ser	47 ± 3	0.4	This work
p.Gly100Asp	42.5 ± 1.6	2.1	This work
IlePheGlyAsp	49 ± 1	2.8	This work
p.Phe225Leu	50.7 ± 2.1	0.6	This work
Q_o domain: proton pathway			
	bc$_1$ complex activity (s^{-1})		
p.Tyr132Phe	23 ± 0.5		This work
p.His253Glu	5 ± 0.3		This work
p.Gly137Arg	17 ± 0.9		This work
p.His253Asp	79.3 ± 5.3		This work
p.His253Glu	85 ± 6		This work
p.His253Asn	78 ± 5		This work

The mutant strains were constructed by biolistic transformation method as described in Materials and Methods, except mutant G137E that was obtained after random mutagenesis (Tron and Lemesle-Meunier 1990). [a] p.Gly143Ala was studied in bacteria *bc*$_1$ complex.

The bc_1 complex activity assays (decylubiquinol cytochrome *c* reduction) and determination of inhibitor mid-point titration (IC_{50}) were performed as described in Materials and Methods. For the first six mutants of the table, the data were from the listed publications.

Supp. References

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III. Discussion et perspectives

Nous avons étudié une série de polymorphismes observés chez l'Homme en utilisant la levure. Les résultats de cette étude les plus intéressants d'un point de vue médical est la modification de la sensibilité à l'atovaquone ou à la clomipramine causée par certaine mutations, en particulier par les mutations en position 172 et 17 (séquence levure).

La plupart des polymorphismes humains étudiés ici sont de rares variations, excepté D171N et F18L (séquence humaine), qui sont fréquemment observés. D171N est une signature de l'haplogroupe J.

D'après notre étude dans la levure, D171N pourrait rendre le complexe bc_1 plus sensible à l'atovaquone. D'autres polymorphismes de ce résidu, S et G, résulteraient dans la même augmentation de la sensibilité. Nous avons étudié l'effet de variation du résidu 172 (levure) introduit dans le site Q_o type levure et dans le site Q_o « humanisé » (résultant de la modification de 8 résidus). Dans le deux cas, N augmente la sensibilité à l'atovaquone tandis que D la diminue. Il faudrait pouvoir tester l'effet de ces variations sur des cellules humaines pour confirmer nos observations. Mais, comme je l'ai mentionné dans l'introduction de ce point, il est très difficile d'obtenir des données biochimiques sures à cause de variations génétiques entre lignées cellulaires existantes et de l'impossibilité de créer, par modification génétique, des lignées portant la ou les mutations dans le cytochrome *b* à étudier.

Pour cela, la levure reste un outil utile, spécialement si nous modifions les sites catalytiques pour les rendre plus semblables à ceux de l'enzyme humaine. Avec l'avancement du séquençage haut débit, il y aurait de plus en plus de variations découvertes dans le cytochrome *b* ou d'autres sous-unités des complexes d'OxPhos à caractériser.

Diverses molécules qui ciblent le complexe bc_1 sont déjà sur le marché ou en cours de développement pour traiter des maladies humaines, animales ou végétales. La levure pourrait donc être un outil pour étudier la sensibilité du complexe bc_1 dans un contexte bien contrôlé. Des conclusions claires sur l'effet de variations sur la sensibilité pourraient être obtenues. Ces données biochimiques pourraient aider à orienter le traitement de certaines maladies pour chaque patient en fonction de leurs variations dans le complexe bc_1. Les doses à prescrire pourraient variés alors pour réduire les effets nocifs et/ou pour augmenter l'efficacité du traitement.

Chapitre II. Études du site Q_i

Chapitre II. Études du site Q_i, construction de souches « *Plasmodium*-like » et mode de liaison d'Endochin-like Quinolones (ELQ)

L'atovaquone, la seule molécule sur marché à action antipaludique ciblant le complexe bc_1, se lie dans le site Q_o. Malheureusement, de nombreux cas de résistance due à des mutations du site Q_o ont été reportés. Il est donc nécessaire de développer d'autres molécules ciblant de site Q_i qui permettraient de contourner ce problème. Une bithérapie avec deux molécules ciblant le site Q_o et l'autre site Q_i serait efficace, car la possibilité de développer simultanément deux mutations de résistance dans les deux sites semble très faible. Les ELQ (Endochin-Like Quinolones) développées dans le laboratoire de M. Riscoe (Portland, USA) sont particulièrement intéressantes. Ces molécules bloquent efficacement la prolifération de *Plasmodium* en inhibant le complexe bc_1 du parasite, et plusieurs se lieraient dans le site Q_i (J. S. Doggett *et al.* 2012) (Allison M. Stickles, de Almeida, *et al.* 2015) Une étude de biothérapie en combinant atovaquone et ELQ-300 (inhibiteur du site Q_i) a été récemment publiée (Allison M. Stickles *et al.* 2016). Dans le modèle murin, cette combinaison semble être très efficace, même envers des parasites portant une mutation de résistance à l'atovaquone.

Dans la première étude, j'ai analysé le mode d'action de l'ELQ-400 qui se lierait simultanément aux deux sites Q_o et Q_i.

Pour étudier ces nouvelles molécules ciblant le site Q_i et mieux comprendre le mode de liaison et le développement de résistance, la reconstruction du site Q_i de l'enzyme de levure en « version *Plasmodium* » serait utile. Ce travail est en cours de réalisation. Dans un premier temps, nous avons remplacé quatre résidus du domaine Q_i (M221, F225, K228 et V223 du cluster 3 présenté dans la Figure 18) par les équivalents de *Plasmodium*. Le mutant obtenu, appelé MFKV, présente un phénotype intéressant que je vais décrire dans la deuxième étude.

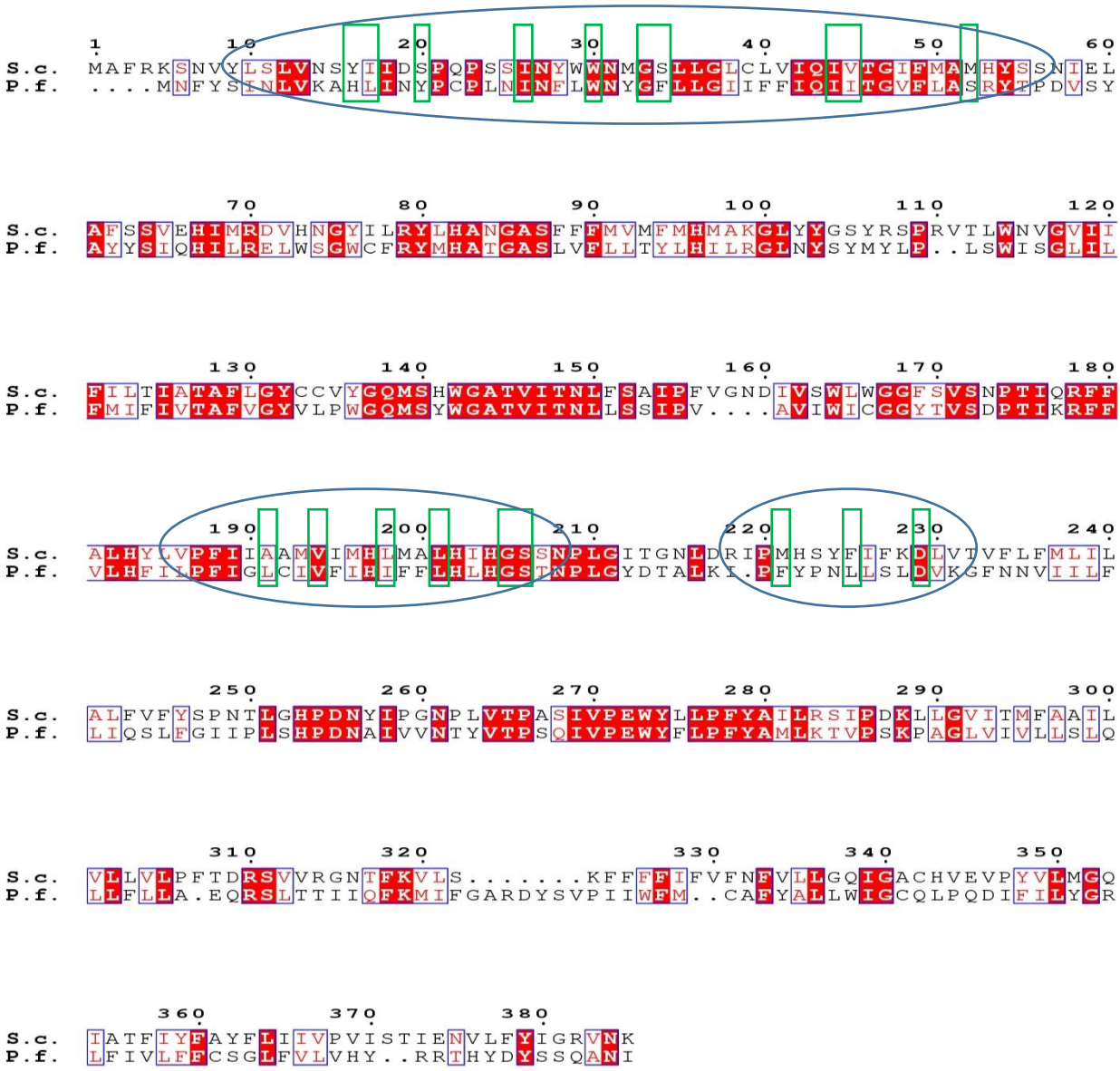

Figure 18. Alignement de séquence du cytochrome *b* de la levure et de *Plasmodium*.
S. c. *Saccharomyces cerevisiae*, P. f. *Plasmodium falciparum*. Les résidus indiqués par les rectangles verts sont les résidus localisés dans un rayon de 5Å par rapport au substrat ubiquinol, donc les résidus qui forment le site Q_i. Les résidus surlignés en rouge sont des résidus bien conservés entre ces deux espèces. Les résidus écrits en rouge sont des résidus qui ont des propriétés physico-chimiques très similaires, donc partiellement conservés. Les rectangles indiquent des motifs de conservation. Les résidus en noir sont des résidus non conservés. Le point noir dans la séquence représente un décalage d'une séquence par rapport à une autre. Les 3 ovales groupent les rectangles verts en 3 clusters formateurs du site Q_i.

Chapitre II-A. ELQ-400, un inhibiteur à double cible

I. Introduction

Les ELQs (Endochin-like quinolone) sont des inhibiteurs du complexe bc_1, développés par le laboratoire de M. Riscoe (Portland, USA). Bien que les ELQs partagent le même pharmacophore et sont tous plus ou moins efficaces pour inhiber le complexe bc_1 du parasite, ils peuvent avoir des sites de liaison différents. Par exemple, ELQ-271 cible le site Q_i tandis que ELQ-121 est un inhibiteur du site Q_o (Allison M. Stickles, de Almeida, *et al.* 2015). De plus, certains ELQs inhibent efficacement le complexe bc_1 de la levure tandis que d'autres sont peu ou pas efficaces. De petites différences structurales entre les ELQs et entre les sites catalytiques de l'enzyme de levure et du parasite pourraient expliquer ces différences d'affinité.

L' étude d'ELQ-400 chez *Plasmodium* a montré que cette molécule est prometteuse (A M. Stickles *et al.* 2015). ELQ-400 est effective contre *Plasmodium* dans le sang et dans le foie. La molécule a une bonne solubilité, et avec une seule dose, elle est capable de réduire rapidement la parasitémie. Elle est efficace contre la souche résistante à l'atovaquone (portant la mutation Y279S dans le site Q_o du complexe bc_1). L'étude a montré qu'il est difficile de sélectionner des parasites résistants à ELQ-400. De plus, l'inhibiteur est peu toxique pour les cellules humaines.

La cible précise d'ELQ-400 reste à déterminer. La molécule pourrait se lier dans le site Q_o ou le site Q_i. Elle pourrait aussi avoir une double action, ciblant à la fois Q_i et Q_o.

La majorité des inhibiteurs ciblent un seul site et sont classifiés en inhibiteurs du site Q_o ou inhibiteurs du site Q_i (Gao *et al.* 2003). Les mutations de résistance obtenues chez des organismes modèles, comme la levure, ou des pathogènes confirment la classification.

Cependant, l'analyse structurale a montré que certains inhibiteurs comme le NQNO et l'ascochlorin sont capables de se lier dans les deux sites (Gao *et al.* 2003) (E. A. Berry *et al.* 2010). Néanmoins, des mutations de résistance au NQNO n'ont été trouvées qu'au site Q_i, ce qui classifie le NQNO comme un inhibiteur de site Q_i (Van Ark and Berden 1977) (von Jagow and Link 1986). Aucune analyse de mutation de résistance à l'ascochlorin n'a été reportée.

Pour tenter de déterminer le site de liaison d'ELQ-400 et identifier les résidus impliqués dans la liaison, nous avons effectué une étude mutationnelle et computationnelle.

Nous avons effectué une analyse de docking *in silico* pour mieux comprendre son interaction avec les deux sites catalytiques. Afin de pouvoir comparer les résultats des dockings avec les résultats de l'étude mutationnnelle, nous avons préparé les fichiers de la structure en introduisant les mutations étudiées. La cardiolipine et quelques molécules d'eau qui sont spatialement bien conservées entre différentes structures publiées ont été gardées, ainsi que l'hème b_H. La structure 3D de la molécule ELQ-400 a été générée. Les dockings ont été réalisés en utilisant le logiciel GOLD.

ELQ400, a dual Q_o and Q_i site inhibitor of the bc_1 complex

Zehua Song [a], Bogdan I. Iorga [b] and Brigitte Meunier [a]

[a] Institute for Integrative Biology of the Cell (I2BC), CEA, CNRS, Université Paris-Sud, Université Paris-Saclay, 91198, Gif-sur-Yvette, France

[b] Institut de Chimie des Substances Naturelles, CNRS, UPR 2301, Labex LERMIT, 91198 Gif-sur-Yvette, France

Corresponding authors:

Bogdan I Iorga, Institut de Chimie des Substances Naturelles, CNRS, UPR 2301, Labex LERMIT, 91198 Gif-sur-Yvette, France

Bogdan.IORGA@cnrs.fr

Brigitte Meunier Institute for Integrative Biology of the Cell (I2BC), CEA, CNRS, Université Paris-Sud, Université Paris-Saclay, 1 avenue de la Terrasse, 91198 Gif-sur-Yvette, France

Tel 00 33 1 69823156

brigitte.meunier@i2bc.paris-saclay.fr

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Short title: *Plasmodium*-like version of yeast bc_1 complex Q_i site

Abbreviations used: Q_o, quinol oxidation site; Q_i, quinone reduction site; Cyt *b*/*c*, cytochrome *b*/*c*; ISP, Iron-Sulfur Protein; OD, optical density; DQ, decylubiquinol; IC_{50}, inhibitor concentration required to obtain 50% inhibition

Summary

The bc_1 complex of the mitochondrial respiratory chain is essential for *Plasmodium falciparum* proliferation, the causative agent of human malaria. The enzyme is thus an attractive target for antimalarials. To date, only the bc_1 complex Q_o site inhibitor atovaquone is used in malaria treatment. However, acquired resistance mutations in the drug binding site rapidly evolve upon treatment, leading to therapeutic failure. New bc_1 complex inhibitors with different binding modes are needed. Endochin-like quinolones (ELQs) are promising molecules, with potentially therapeutic actions in different domains, including malaria. Among them, ELQ400 is a potent inhibitor of *P. falciparum* proliferation, and still active against atovaquone resistant parasite. Here we used a yeast model of *P. falciparum* bc_1 complex to investigate the drug mode of action. Analysis of a series of yeast mutants strongly suggested that ELQ400 targets both sites, as both mutations in Q_i and Q_o sites resulted in increased sensitivity to the drug. *In silico* modelling of ELQ binding in the catalytic sites revealed possible stabilising interactions with residues in both Q_i and Q_o sites.

Introduction

The mitochondrial respiratory chain complex III or bc_1 complex is central to mitochondrial bioenergetics and the target of antiprotozoals, such as atovaquone, and fungicide drugs used to control human and plant pathogens. The bc_1 complex is a multimeric enzyme, with three subunits forming the catalytic core that contain the redox-active groups (haems and [2Fe-2S]), *i.e.* two cytochromes *b* and c_1 and the iron-sulfur protein (ISP). The crystal structures of several bc_1 complexes from different species have been solved. These structures share the same catalytic core, with cytochrome *b* as the central and largest subunit, a predominantly hydrophobic protein consisting of eight transmembrane helices. Cytochrome *b* is mitochondrially encoded in all eukaryotes, while the other subunits are nuclearly encoded.

The bc_1 complex catalyses the transfer of electrons from ubiquinol to cytochrome *c* and couples this electron transfer to the vectorial translocation of protons across the inner mitochondrial membrane, contributing to the protonmotive force used for ATP synthesis by the ATP synthase. The catalytic mechanism, called the Q-cycle, bifurcates electron flow across high- and low-potential electron transfer chains within the bc_1 complex, and requires two distinct quinone-binding sites (Q_o, quinol oxidation site, and Q_i, quinone reduction site), which are located on opposite sides of the membrane and linked by a transmembrane electron-transfer pathway, reducing one molecule of ubiquinone per two molecules of ubiquinol oxidised. Cytochrome *b* provides both the Q_o and Q_i pockets and the (low potential) transmembrane electron pathway (*via* haems b_L and b_H). The high potential chain from Q_o to cytochrome *c* is formed by the ISP and cytochrome c_1.

Important pathogens, such as the malaria parasite *Plasmodium falciparum*, but also *Toxoplasma gondii* and *Babesia microti* are heavily dependent on the bc_1 complex function for cell maintenance and proliferation. The respiratory enzyme is thus a good target for anti-microbial drugs.

To date, atovaquone (in combination with proguanil) is the sole bc_1 complex inhibitor used as anti-microbial drug. The compound tightly binds in the Q_o site of the complex (Birth, Kao, and Hunte 2014), efficiently blocking the catalytic activity. Unfortunately, numerous cases of resistance have been reported in pathogens isolated from patients after treatment failure. This resistance is mostly linked to mutations within the Q_o site. In *P. falciparum* responsible of the most serious cases of Malaria, amino acid substitutions of Y268 (Y279 in yeast), Y268C and Y268S, are frequently observed. The mutations cause a high level of resistance as also observed in yeast (Song, Clain, Iorga, Yi, *et al.* 2015). Therefore, new

drugs that target the bc_1 complex but that can circumvent atovaquone resistance are needed. Among the different compounds that are currently being studied, of particular interest are the ones binding in the Q_i site (see for instance, (J Stone Doggett *et al.* 2012) (Allison M. Stickles, Justino de Almeida, *et al.* 2015) (Capper *et al.* 2015) (Vallières *et al.* 2012).

Endochin-like quinolones (ELQs) are potent inhibitors of the parasite's bc_1 complex (A. Nilsen *et al.* 2014) (R. Winter *et al.* 2011) (R. W. Winter *et al.* 2008). ELQ300, for instance, is highly active against liver, blood and transmission stages of the parasite and demonstrates a remarkable efficacy against atovaquone resistance parasite (A. Nilsen *et al.* 2013). *P. falciparum* clones resistant to ELQ300 were obtained after selection, in which the mutation I22L (residue I26 in yeast), located in the Q_i site, was observed. This strongly suggests that ELQ300 targets the Q_i site (Allison M. Stickles, de Almeida, *et al.* 2015).

ELQ400 was also reported as active against parasites at key stages (A M. Stickles *et al.* 2015). The drug is effective against atovaquone resistance *P. falciparum* strains but to a lower extent than ELQ300. In addition, ELQ300 resistance mutation I22L did not result in cross-resistance to ELQ400, suggesting a different mode of action (Allison M. Stickles, de Almeida, *et al.* 2015).

In this study, we used yeast as a model to investigate the target of ELQ400. Yeast can be a useful surrogate model as cytochrome b amino acid sequences, in particular at Q_o and Q_i sites, are highly conserved between organisms. In addition, yeast is amenable to mitochondrial transformation and site-directed modification of mtDNA genes, such as the cytochrome b gene. We tested the effect of single or multiple amino-acid substitutions in the catalytic sites Q_o and Q_i on the sensitivity to ELQ400. In parallel, *in silico* models of the Q_o and Q_i modified sites were built to predict, using molecular docking, the interaction of ELQ400 with the catalytic sites.

2 Materials and Methods

2.1 Materials and growth media

Equine cytochrome *c*, decylubiquinone, azoxystrobin, antimycin, superoxide dismutase, and catalase were obtained from Sigma Aldrich. The following media were used for the growth of yeast: YPD (1% yeast extract, 2% peptone and 3% glucose), YPGal (1% yeast extract, 2% peptone and 2% galactose), YPeth (1% yeast extract, 2% peptone and 3% ethanol) and YPG (1% yeast extract, 2% peptone and 2% glycerol).

2.2 Yeast strains

The multiple mutations were introduced in yeast cytochrome *b* by side-directed mutagenesis and mitochondrial transformation (Hill *et al.* 2003) (Nicholas Fisher, Castleden, *et al.* 2004). In all experiments, control and mutants have identical nuclear and mitochondrial genomes with the exception of the mutations introduced in the cytochrome *b* gene.

Growth experiments were performed using strain AD1-9 lacking several membrane transporters (*a ura3 his1, yor1⊿ ::hisG, snq2⊿::hisG, pdr5⊿:: hisG, pdr10⊿ ::hisG, pdr11⊿ ::hisG, ycf1⊿ ::hisG, pdr3⊿ ::hisG, pdr15⊿ ::hisG, pdr1⊿ ::hisG*) (kindly provided by M. Ghislain, UCL, Belgium).

2.3 Measurement of decylubiquinol-cytochrome *c* reductase activity

Yeast mitochondria were prepared as in (Lemaire and Dujardin 2008). Briefly, yeast grown in YPGal medium were harvested at mid-log phase. Protoplasts were obtained by enzymatic digestion of the cell wall using zymolyase in an osmotic protection buffer. Mitochondria were then prepared by differential centrifugation following osmotic shock of the protoplasts. Mitochondrial samples were aliquoted and stored at -80 °C. Concentration of bc_1 complex in the mitochondrial samples was determined from dithionite-reduced optical spectra, using ε=28.5 mM^{-1} cm^{-1} at 562 nm *minus* 575 nm. Decylubiquinol-cytochrome *c* reductase activities were determined at room temperature by measuring the reduction of cytochrome *c* (final concentration of 20 µM) at 550 nm *versus* 540 nm over 1-min time-course in 10 mM potassium phosphate pH 7, 0.01% (w/v) lauryl-maltoside and 1 mM KCN. Mitochondria were added to obtain a final concentration of 5-30 nM bc_1 complex. Activity was initiated by the addition of decylubiquinol, a synthetic analog of ubiquinol (final concentrations of 2.5-60 µM). Initial rates were measured as a function of the decylubiquinol concentration. Each measurement was repeated three to five times and the values obtained were averaged. The mid-point inhibition concentrations (IC_{50}) were determined by inhibitor titration.

2.3. Ligand docking and molecular modelling

Three-dimensional structure of ELQ-400 was generated using CORINA (Molecular Networks GmbH, Erlangen, Germany, http://www.molecular-networks.com). The structure of the yeast bc_1 complex 3CX5.pdb was downloaded from the Protein Data Bank (Berman *et al.* 2000) and used as receptor in the docking process. Water molecules in binding sites and haems are kept. Ligands, lipids and water molecules outside the two binding sites were removed, as well as all other chains except those close to cytochrome *b* and ISP. Hydrogen atoms were added using HERMES, the graphical interface of GOLD (Verdonk *et al.* 2003). The docking of ELQ-400 was performed with GOLD using a binding site defined as a 20Å radius sphere centered on the δ1-carbon atom of cytochrome *b* residue I147 (Q_o site) and a 20Å radius sphere centered on the γ-oxygen atom of cytochrome b residue S206 (Q_i site). "Toggle" function for water molecule was activated. GoldScore was used as a scoring function and all other parameters had default values. *In silico* mutations were introduced using CHIMERA (Pettersen *et al.* 2004), which was also used for generating the molecular modeling images.

Results and discussion

<u>ELQ400 inhibits yeast *bc*1 complex activity</u>

We first tested the inhibitory effect of ELQ400 on yeast wt bc_1 complex activity, measured as decylubiquinol-cytochrome *c* reductase activity. The obtained IC_{50} value (mid-point inhibitory concentration) was compared with the IC_{50} values for four other anti-microbial compounds:

ELQ271 and ELQ316, which are potent inhibitors of *T. gondii* (J Stone Doggett *et al.* 2012) and of *P. falciparum*, the fungicide azoxystrobin and the antimalarial atovaquone. These latter compounds bind at the bc_1 complex Q_o site while ELQ271 targets the Q_i site (Doggett *et al.* 2012). ELQ316 is similar to the lead compound ELQ300, and this is likely to target the Q_i site (A. Nilsen *et al.* 2013)

ELQ400, ELQ271 and ELQ316 differ by the 5-, 6- and/or 7-position substituents (Allison M. Stickles, de Almeida, *et al.* 2015) (Fig.1).

In *P. falciparum* growth assays, ELQ400 and ELQ316 displayed potent inhibitory activities with IC_{50} values of 1.5 and 3.1 nM, respectively, while ELQ271 was slightly less active with an IC_{50} of 12 nM (Allison M. Stickles, de Almeida, *et al.* 2015).

By contrast, ELQ316 was found to be inactive against yeast bc_1 complex. ELQ400 displayed a lower reactivity compared to ELQ271 and to the Q_o site inhibitors (Table 1).

Table 1: Inhibitory effect of ELQs on wt bc_1 complex activity

Inhibitor	IC_{50}/[bc_1]
ELQ400	88 ± 7.2
ELQ271	15 ± 2
ELQ316	>350
Azoxystrobin	12 ± 1.2[a]
Atovaquone	4 ± 0.6[a]

The bc_1 complex activity was monitored as described in Materials and Methods. IC_{50} values were determined by inhibitor titration and reported on bc_1 complex concentrations. [a] from (Song, Clain, Iorga, Yi, *et al.* 2015)

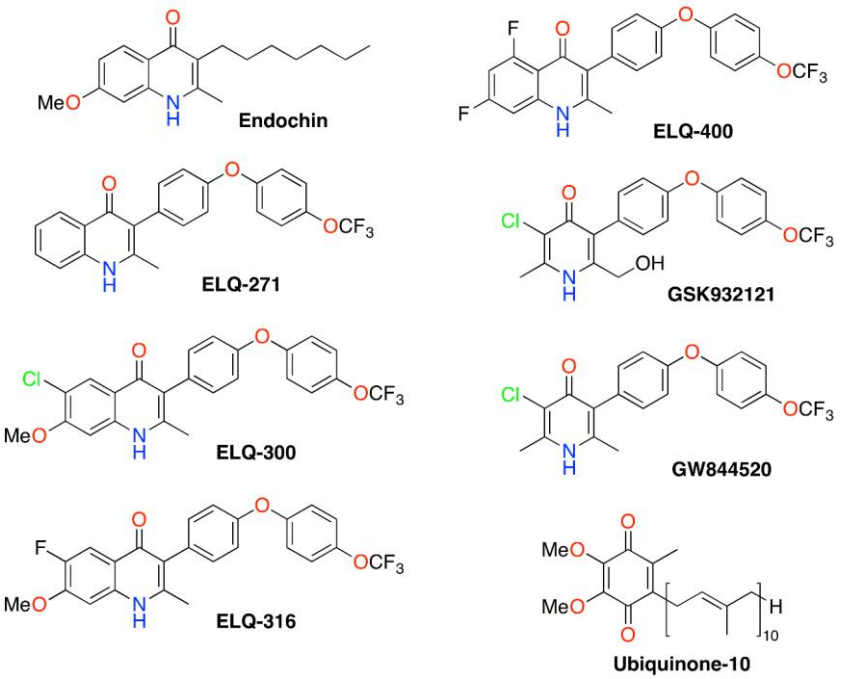

Figure 1. Chemical structures of Endochin, ELQ271, ELQ-316, ELQ-300, ELQ-400, pyridone GSK932121 and GW844520

In parallel, we monitored the inhibitory effect of ELQ271 and ELQ400 on yeast respiratory growth, using a strain lacking several membrane transporters, which renders the cells more sensitive to added compounds in the growth medium, such as atovaquone (Hill *et al.* 2003). The wt strain was cultivated in YPEth medium with increasing concentration of inhibitors. Growth yields (measured as OD600 nm after three days of culture) were then recorded. We observed that ELQ271 was a potent inhibitor of yeast respiratory growth as the growth yield at 0.2 µM ELQ271 reached only 1% of the growth yield of the untreated culture. This is similar to the inhibitory effect of atovaquone. By contrast, no growth inhibition was observed at 10 µM ELQ400. It seems therefore than ELQ271 and ELQ400, despite very similar chemical structures, are not transported in and out the yeast cell in the same manner. More work would be required to identify membrane transporter presumably causing ELQ400 efflux.

Effect of Q_i and Q_o sites mutations on ELQ400 inhibition

We previously showed that, in yeast, the Q_i site mutation M221Q conferred resistance to ELQ271 (J Stone Doggett *et al.* 2012), and this result was confirmed in the present study. We monitored the bc_1 complex activity of M221Q in presence of ELQ271 and obtained an IC_{50} of 262 ± 13, compared to 15 ± 0.4 for the wt.

We then tested the inhibitory activity of ELQ400 on the bc_1 complex activity of M221Q and of a series of additional mutants, harbouring single or multiple amino-acid substitutions in the catalytic sites.

At the Q_i site, M221F, F225L, K228L, and the double mutation F225L-K228L replaced yeast residues by the *P. falciparum* equivalents; the substitution K228I is similar to the *Plasmodium*-like K228L.

At the Q_o site, PF2, PF8 and PF-Y279 result from the substitutions of several yeast residues by the *P. falciparum* residues. PF-Y279S combines the ten *Plasmodium*-like residue replacement of PF-Y279 with the atovaquone resistance mutation Y279S (Y268S in *P. falciparum*) (Vallières, Fisher, and Meunier 2013) (Song, Clain, Iorga, Vallières, *et al.* 2015).

Table 2. Effect of Q_i and Q_o site mutations on ELQ400 sensitivity

	IC_{50}/[bc_1] to ELQ400
wt	88 ± 7.2
Q_i site mutations	
F225L	17 ± 1.2
M221Q	60 ± 5.1
M221F	69 ± 5.9
K228L	36 ± 8.1
K228I	28 ± 5.6
F225L, K228L	37 ± 4.2
Q_o site mutations	
PF2 $CCVY_{133\text{-}136}$VLPW, H141Y, L275F	85 ± 8
PF8 L275F, R283K, MF295-296VL, I299L	15 ± 2
PF-Y279 $CCVY_{133\text{-}136}$VLPW, H141Y, L275F, R283K, MF295-296VL, I299L	5 ± 0.1
PF-Y279S $CCVY_{133\text{-}136}$VLPW, H141Y, L275F, Y279S, R283K, MF295-296VL, I299L	21 ± 4.0

The bc_1 complex activity was monitored as described in Materials and Methods. IC_{50} values were determined by inhibitor titration and reported on bc_1 complex concentrations.

As shown in Table 2, M221Q and M221F had no or very minor effect on ELQ400 sensitivity. Thus, M221Q did not confer cross resistance ELQ271 and ELQ400.

Replacement of large yeast residues F225 and K228 by the smaller *Plasmodium*-like residue leucine increased two to five fold the sensitivity to ELQ400. The double mutation F225L-K228L and the mutation K228I resulted in the same effect. The mutations might increase the accessibility of the drug to the Q_i site or slightly stabilize its binding.

Interestingly, modification of the Q_o site also resulted in an increased sensitivity. PF-Y272, with ten *Plasmodium*-like changes displayed a 17-fold increased sensitivity with an IC_{50} for ELQ400 similar to the IC_{50} for atovaquone. The five changes of PF8 also resulted in 6-fold increase in sensitivity, whereas the six changes of PF2 had no effect on ELQ400 sensitivity.

It seems therefore that ELQ400 accessibility and/or stabilisation into the Q_o pocket is enhanced by the *Plasmodium*-like modifications. Mutation at positions 283, 295, 296 and 299 would have the greatest impact.

Residue Y279 is also likely to be involved in ELQ400 binding as the introduction of the atovaquone resistance mutation Y279S in PF-Y279 caused a 4-fold decrease in ELQ400 sensitivity (IC_{50} increasing from 5 to 21 for PF-Y279 and PF-Y279S, respectively).

This result is in agreement with the data obtained with *P. falciparum* growth assays. The sensitivity to ELQ300, ELQ316 and ELQ271 was unchanged in the atovaquone resistance parasite compared to control, while the ELQ400 resistance was 20-fold increased (Allison M. Stickles, de Almeida, *et al.* 2015). However, this was a moderate increase in resistance compared to the major >3000-fold increased resistance to atovaquone.

Mutational analysis in yeast suggests that ELQ400 could target both Q_o and Q_i sites, as mutations in either sites increased the drug sensitivity of the bc_1 complex. *P. falciparum* has been found to be highly sensitive to ELQ400. Interestingly, the mutations that replaced yeast residues by the *Plasmodium* equivalents rendered the yeast enzyme more sensitive to the drug.

In silico models of ELQ400 bound in the Q_o and Q_i sites

Q_i site

Our docking analysis suggested that ELQ-400 would bind in the core of the Q_i site with its aromatic headgroup in a similar way than the substrate ubiquinol (Solmaz and Hunte 2008) (Birth, Kao, and Hunte 2014) and of pyridones (Capper *et al.* 2015). The structures of the compounds are shown in Fig.1. The bicyclic systems would be aligned and they would share the same interactions with the binding pocket. The compounds GW844520 and GSK932121 and ubiquinone have only one ring on the headgroup, while ELQ-300 and ELQ-400 have two rings. As observed in the yeast X-ray crystallographic structures, there is little space leftover in the core Q_i binding pocket, and pyridones and ubiquinone's headgroups already touch the bottom of the binding pocket. ELQ-400, with its bicyclic system bearing small fluorine substituents, may manage to push the sidechains of surrounding residues and accommodate into the binding site. ELQ-300 and ELQ-316 have additional bulky substituents (methoxy and chlorine, see Fig.1), which do not allow them to fit in yeast Q_i site. *Plasmodium* Q_i site that can accommodate ELQ-300 would be larger that yeast Q_i site. This would explain the differential sensitivity of yeast and parasite bc_1 complex to that drug.

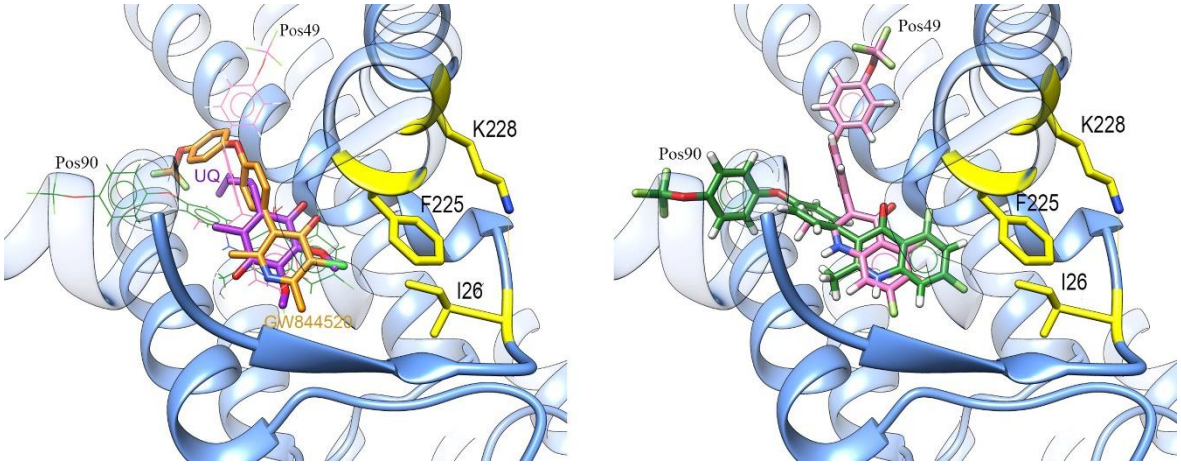

Figure 2. The position of ELQ-400 revealed by docking analysis show similarities to the binding positions of GW844520 and ubiquinone-10. Left, positions of GW844520 and ubiquinone (PDB code: 4D6T and 2A06, respectively). Right, the two best positions for ELQ-400 obtained by molecular docking.

In yeast, the mutation F225L, which induces a higher sensibility to ELQ-400, introduces a smaller side chain of leucine compared to those of phenylalanine. This mutation leaves enough space for the sidechain of I26, which is in direct contact with the aromatic ring of the inhibitors (and substrate), to rotate and create more space for ELQ-400 in the Q_i site.

Residue K228 seems to play an important role in the flexibility of the helix E and A that surround the Q_i site. A hydrogen bond is present between the sidechain of K228 (helix E) and the backbone of S28 (helix A). When the lysine is replaced by a leucine, this hydrogen bond is lost and the flexibility between helix E and A would increase, which may help to create more space for the Q_i binding site. By consequence, ELQ-400 would have more space for binding, which explains the increased sensitivity of the mutant.

Q_o site yeast PF mutations

Our docking analysis suggested that the Endochin-like moiety of ELQ-400 would be in a similar position to the aromatic rings of stigmatellin and atovaquone (Fig.3). The software predicts a hydrogen bond between H181 of ISP and the NH group of ELQ-400. This group acts as a hydrogen bond donor, like the hydroxyl group of stigmatellin and atovaquone. Even though no available structure of the *bc*$_1$ complex shows how the substrate binds inside the Q_o site, probably because of its mobility inside the Q_o site, it is well accepted that at least in one configuration, the headgroup of the substrate should bind similarly to that of stigmatellin and atovaquone. Analogously, ELQ-400 would also mimic the substrate binding.

It is likely that ELQ-400 binding in the Q_o site would be stabilized by its interaction with Y279. The mutation Y279S would then result in a (perhaps major) loss of stabilization. However the observed resistance resulting from the introduction of Y279S was minor (5-fold increase, Table 2). This can be explained by the unchanged mode of binding of the drug in the Q_i site, and the differences would be the effect of small changes in the interaction with the surrounding residues.

Examination of the structures suggested that the increased sensitivity of PF-Y279 as compared to wt could be explained by a slightly increased volume of the mutated Q_o site, making more room for the accessibility and binding of the bulky inhibitor. In this way, the inhibitor can have a facilitated access to establish a hydrogen bond with the side chain of H181.

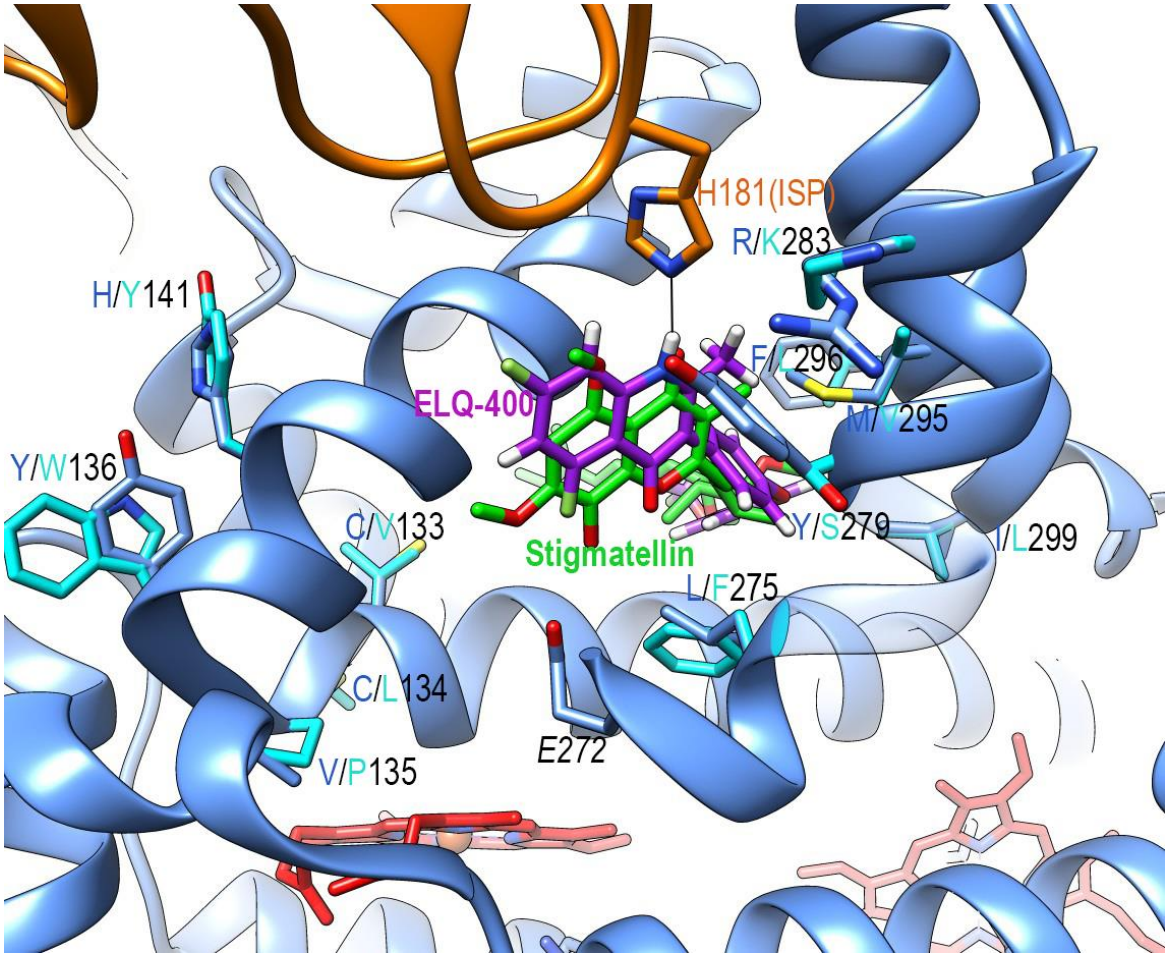

Figure 3. Co-localization of ELQ-400 and stigmatellin in the Q_o site. Cytochrome b is colored in blue, ISP in orange and haems in red. The hydrogen bond with His181 is drawn with a black line and shared by the stigmatelline and the docked ELQ-400. The positions of the mutations of PF-Y279 and PF-Y279S are shown (cyan). The first letter of each label is the wt residue (blue); the second one is the substitution (cyan).

Plasmodium resistance to ELQ-400 induced by mutation V270L in the Q_o site

As reported in a recent study (A M. Stickles *et al.* 2015), parasites resistant to ELQ400 were obtained after selection. The mutation V259L (V270 in yeast) was observed, which conferred a 10-fold increase resistance to ELQ400 and atovaquone. The mutation is located in the Q_o site region (Fig.4). The side chain of amino acid 270 is outside the Q_o binding side, but the loop *ef* containing the residue 270 is flexible. A water molecule might be present in the *Plasmodium* structure between the side chains of residues C334 (A341 in yeast) and Q257 (S268 in yeast) and forms hydrogen bonds with both amino acids. This would stich helix G (319-342) to the loop *ef* (245-274).

Residue 270 doesn't interact directly with bound atovaquone or ELQ-400. However, in mutant V270L, the larger sidechain of leucine might clash with this water molecule-mediated connection between helix G and loop *ef*. Comparison of published structures shows that at least two conformations are possible for the loop *ef* around the residue 270 (fig 4). One makes the Q_o site smaller than the other. In the "smaller Q_o site" conformation, I269 (also on *ef* loop) that points towards the Q_o site might push the inhibitor outside of the binding pocket, making it less efficient. Thus it is possible that V270L clashing with the connection between C334 and N257 would be pushed back and, as a result, the loop would take the conformation which forms a smaller Q_o site. This could lead to a less adapted Q_o site for atovaquone and ELQ-400 and would explain the moderate resistance observed in *Plasmodium*.

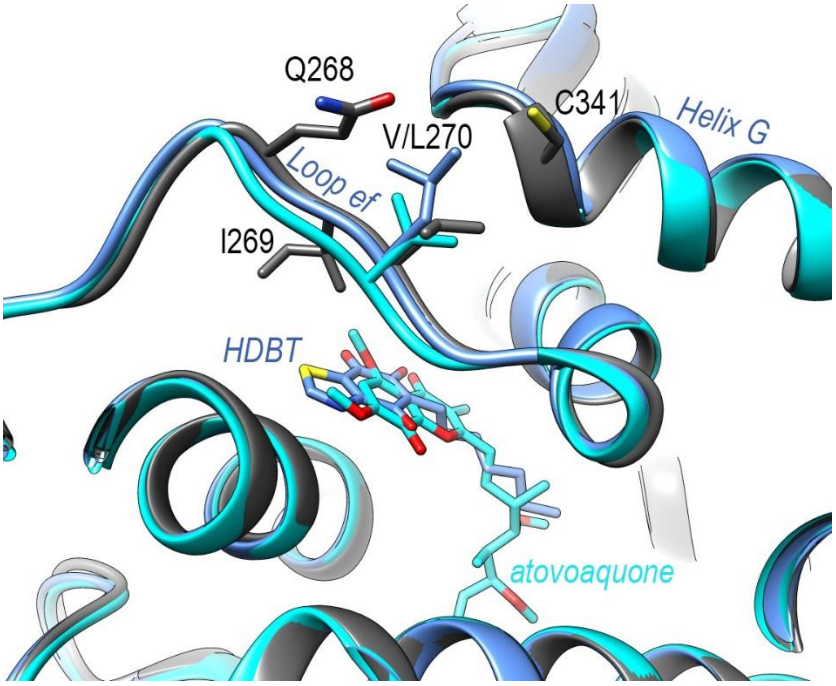

Figure 4. Location of residue 270 in the Q_o binding region with bound inhibitor atovaquone and HDBT. In blue and cyan, yeast cytochrome b crystallographic structures (PDB codes: 1P84 and 3CX5, respectively). In dark gray, *Plasmodium* cytochrome *b* structure modeled on the basis of yeast 4PD4 structure. The mutation V270L has been introduced in one of the yeast structures (Blue).

Conclusion

It was previously hypothesized that some of the ELQs could target simultaneously the two binding sites of the bc_1 complex (Allison M. Stickles, de Almeida, *et al.* 2015). The data obtained here with yeast mutants validate this hypothesis and clearly indicated that indeed ELQ400 target both Q_o and Q_i sites.

Most bc_1 complex inhibitors bind to either Q_o or Q_i sites, but not to both. The structural differences between the two sites make unlikely the tight binding of a compound in both sites. NQNO that structurally resembles the substrate quinol/quinone is able to bind at both sites but with a relatively low affinity to either site ((Gao *et al.* 2003) and refs within). Yeast NQNO resistant mutants were selected and mutations were only found in the Q_i site. Among them, the mutations M221Q and F225L were studied in the present work. Therefore, NQNO is reported as a Q_i site inhibitor. Ascochlorin also binds at both catalytic sites of the complex, as observed on bacterial and animal bc_1 complexes (E. A. Berry *et al.* 2010). However no resistance mutations have been analysed.

Examination of the substrate-bound and inhibitor-bound structures and of ELQ-400 docking models suggested that the ligands have similar positions and similar interactions with the binding pockets, both at the Q_o and Q_i site.

If ELQ-400, as suggested, has a binding mode similar to the substrates, it could be expected that mutations that severely compromise its affinity might also decrease the affinity of substrate to the catalytic sites. Resistance mutations would then resulting in a lower bc_1 activity and compromised proliferation, as it was observed for atovaquone resistance.

Interestingly, it has been recently reported that parasites harbouring atovaquone resistance mutations could not be transmitted to mosquitoes because of the less active enzyme (Goodman *et al.* 2016). We might expect the same phenomenon for the mutations of resistance to ELQ-400. In addition, dual-site

inhibition would be protective against acquired drug resistance, as high-level resistance would require the simultaneous appearance of two mutations in cytochrome b. So far, only a mutation conferring a low level of resistance has been observed after selection (A M. Stickles *et al.* 2015). That level of resistance would probably not compromise the efficacy of the treatment.

In addition, ELQ-400 didn't show toxicity in mice (A M. Stickles *et al.* 2015). These properties makes it a promising drug to carry on the preclinical and clinical research and develop it in a new anti-malarial drug.

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III. Perspectives

Il serait intéressant de poursuivre l'analyse du mode d'action de l'ELQ-400 pour confirmer les données obtenues dans cette étude.

La mutation de résistance à ELQ-400, V270L, dans le site Q_o et les mutations de sensibilisation F225L et K228I/L, dans le site Q_i, ont été analysés de façon statique dans notre étude *in silico*. Il serait intéressant de pousser plus loin leur analyse et de faire une simulation de dynamique moléculaire autour de ces résidus pour mieux évaluer l'effet des mutations sur la conformation des sites et ensuite sur son affinité pour inhibiteurs et substrat.

Les analyses mutationnelle et *in silico* présentées ici indiquent clairement que l'ELQ-400 agit aux deux sites catalytiques. Une approche de spectroscopie permettrait de confirmer cela. Quand le complexe bc_1 est inhibé par l'antimycine et funiculosine, deux inhibiteurs du site Q_i, le signal optique à 562nm est déplacé vers le rouge ou vers le bleu, respectivement. Tandis que quand le complexe est inhibé par stigmatelline et MOA-stilbène, le signal à 566nm est déplacé ver le rouge. Ce serait intéressant de regarder si ELQ-400 pourrait induire un déplacement des deux signaux.

Nous pourrons aussi tester l'effet de l'ELQ-400 sur le potentiel redox des hèmes *b*. Si ELQ-400 se lie de la même façon que la stigmatelline et l'atovaquone dans le site Q_o, sa liaison devrait modifier le signal EPR (Electron Paramagnetic Resonance) du groupe [2Fe-2S] de l'ISP comme le fait la liaison de ces inhibiteurs (Kessl *et al.* 2003) (Nicholas Fisher, Bourges, *et al.* 2004).

Enfin, cette étude des ELQs met en lumière une différence entre les sites Q_o et Q_i qu'il serait intéressant d'explorer.

Le site Q_o présente des affinités différentes selon les espèces envers certains inhibiteurs comme l'atovaquone. L'enzyme mammifère est moins sensible que les enzymes de levure et de *Plasmodium* (Vallières, Fisher, and Meunier 2013). Cette propriété est requise pour le développer de molécules thérapeutiques. Les différences d'affinité entre espèces semblent être bien plus importantes pour le site Q_i. Par exemple, l'ELQ-316, un inhibiteur du site Q_i, est efficace contre *Plasmodium*, mais n'a aucun effet contre la levure. Ceci pourrait être expliqué par le fait que le site Q_i est moins bien conservé que le site Q_o parmi les espèces (*c. f.* l'« Introduction aux problématiques de cette thèse » point B). Il serait intéressant de faire une analyse des séquences et des modèles de structures, et de comparer les deux sites avec un éclairage évolutif.

Chapitre II-B. Le mutant MFKV et un passage de protons

I. Introduction

Le site Q_i est une cible thérapeutique intéressante, et certains antipaludiques en cours de développement se lient dans ce site, par exemple certains ELQs. Pour étudier, dans la levure, le mode de liaison des ces nouveaux inhibiteurs et le développement de mutations de résistance, une enzyme de levure avec un site Q_i modifié, ressemblant davantage au site Q_i de l'enzyme du parasite serait un outil plus adéquat. Cette approche a été utilisée dans l'étude du site Q_o de *Plasmodium* (chapitre I-B).

Le site Q_i est plus grand que le site Q_o, et la variation de séquence d'acides aminés entre la levure et le *Plasmodium* est plus importante. Comme présenté dans la Figure 18, les 19 résidus dans un rayon de 5Å de l'ubiquinol, qui sont considérés comme résidus formateurs du site Q_i, peuvent être groupés en 3 clusters.

Nous avons commencé la reconstruction du site Q_i de levure « façon *Plasmodium* » par l'introduction des modifications dans le 3ème cluster (Figure 18). Quatre acides aminés ont été changés: M221F, F225L, K228L et V233F, et le mutant a été appelé MFKV (Figure 19).

Les résidus M221 et F225 sont deux acides aminés formant le site Q_i (c'est-à-dire localisés dans un rayon de 5A de l'ubiquinone) quoique peu conservés entre les espèces. K228 est un résidu très conservé dans beaucoup d'espèces mais remplacé par une leucine chez *Plasmodium*. K228 est considéré comme un résidu clé pour le passage des protons par la voie Cardiolipine/K, décrite précédemment (chapitre II-A) (Hunte, Palsdottir, and Trumpower 2003). Nous avons aussi introduit la mutation V233F car une phénylalanine, avec sa chaîne latérale beaucoup plus volumineuse que la valine, pourrait introduire un encombrement stérique (Figure 19).

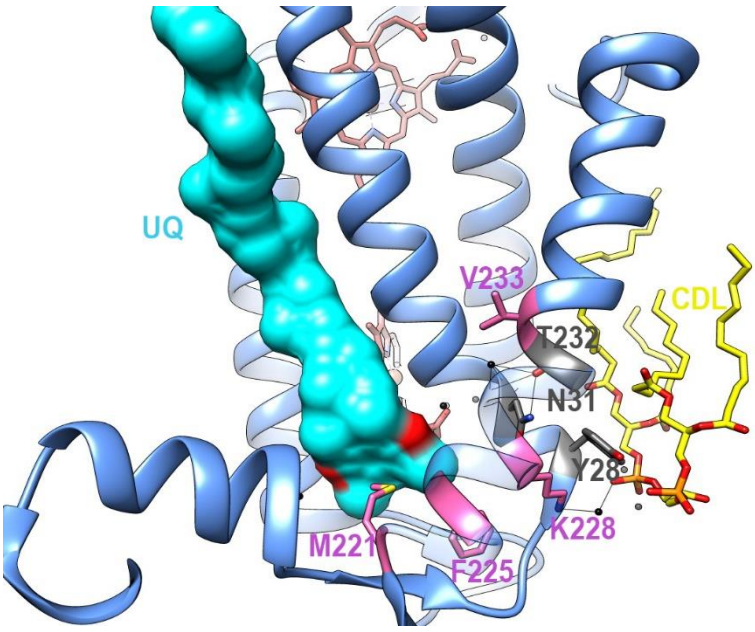

Figure 19. Positions des résidus M221, F225, K228, V233 (rose) ; Y28, N31, F227 et T232 (gris) dans le site Q_i. Le cytochrome *b* (ruban) est en bleu. L'ubiquinone-6 (UQ) (surface) est en cyan ; les deux oxygènes sont colorés en rouge. La cardiolipine (CDL) est en jaune. Les hèmes sont en rouge. Les molécules d'eau sont représentées comme des points noirs. Le résidu K228 et la cardiolipine peuvent former chacune une liaison hydrogène avec la même molécule d'eau. Structure PDB utilisée : 1KB9.

Blocking and unblocking the Q_i site proton entry

Zehua Song [a], Bogdan I. Iorga [b], Nicholas Fisher [c], and Brigitte Meunier [a]

[a] Institute for Integrative Biology of the Cell (I2BC), CEA, CNRS, Université Paris-Sud, Université Paris-Saclay, 91198, Gif-sur-Yvette, France

[b] Institut de Chimie des Substances Naturelles, CNRS, UPR 2301, Labex LERMIT, 91198 Gif-sur-Yvette, France

[c] Plant Research Laboratory, Michigan State University, East Lansing, MI 48824, USA.

Corresponding author:

Brigitte Meunier Institute for Integrative Biology of the Cell (I2BC), CEA, CNRS, Université Paris-Sud, Université Paris-Saclay, 1 avenue de la Terrasse, 91198 Gif-sur-Yvette, France

Tel 00 33 1 69823156

meunier@i2bc.paris-saclay.fr

Keywords: respiratory complex III, yeast model, ROS

Abbreviations used: Q_o, quinol oxidation site; Q_i, quinone reduction site; Cyt *b*/*c*, cytochrome *b*/*c*; ISP, Iron-Sulfur Protein; SO, superoxide ; SOD, superoxide dismutase; OD, optical density; DQ, decylubiquinol; k_{cat}, catalytic activity reported on bc_1 complex concentration; IC_{50}, inhibitor concentration required to obtain 50% inhibition

Summary

The proton pathways have an important role in mitochondrial bc_1 complex. They are channels formed by residues and water molecules which can ensure the translocation of protons at optimal rate. Protons arrive in Q_i site from the matrix via two such channels and are used to produce ubiquinol from ubiquinone. Mutations in the protons pathways that impair the proton translocation may modify the reaction rate in the Q_i site. In this study, we characterized two yeast mutants which have high bc1 activity with no respiratory growth. Several yeast mutants harbouring mutations in or in the vicinity of one of the Q_i site proton pathways, the K228/cardiolipine pathway, restored the respiratory growth. The study showed the key role of this pathway for the catalytic mechanism. We demonstrated that this proton

pathway seems to tolerate modifications and its dysfunction could be restored by supplementary mutation.

Introduction

The mitochondrial respiratory chain complex III or bc_1 complex is a multimeric enzyme. Three subunits form the catalytic core, cytochrome *b*, cytochrome c_1 and the iron-sulfur protein. Cytochrome *b*, central and largest subunit, consisting of eight transmembrane helices, is mitochondrially encoded in all eukaryotes while the other subunits are nuclearly encoded.

The catalytic mechanism, called the Q-cycle requires two distinct quinone-binding sites (the quinol oxidation site, Q_o, and the quinone reduction site, Q_i), which are located on opposite sides of the membrane and linked by a transmembrane electron-transfer pathway. The membrane-spanning cytochrome *b* provides the Q_o and Q_i sites and the transmembrane electron pathway (*via* the two haems bound to this subunit, haems b_L and b_H). A quinol molecule binds at the Q_o -site, is deprotonated, and transfers one electron through the iron-sulphur protein and cytochrome c_1 to cytochrome *c*. The second electron reduces *bL*. It is then transferred to b_H and to a quinol bound at the Q_i site, forming a stable semiquinone species. A second quinol oxidation event at the Q_o site completes the Q-cycle with the formation of fully reduced quinol at the Q_i _site. Overall, two molecules of quinol are oxidised to quinone at the Q_o site and one molecule of quinone is reduced to quinol at the Q_i site.

The oxidation and reduction of quinol/quinone at Q_o and Q_i sites in a hydrophobic environment require pathways for proton movement to and from the aqueous phase. Two short pathways for the uptake of protons form the matrix into the Q_i pocket have been described in the yeast structure, namely the E/R-pathway and the cardiolipine/K-pathway (Hunte, Palsdottir, and Trumpower 2003a). R218 and residue E52 located on the subunit Qcr7, with several water molecules form the E/R pathways. K228, highly conserved in mitochondrial cytochrome *b*, seems the key residue of the cardiolipine/K pathways. K228 connects the water molecules of the Q_i site with the aqueous phase via one water molecule in hydrogen bond distance between K228 and the cardiolipine tightly bound to the protein surface. Structure of the bovine bc_1 complex with bound antimycin in the Q_i site showed that the inhibitor formed a H-bond via a water molecule to K228 (Huang *et al.* 2005).

In addition to antimycin and HQNO, known for decades, inhibitors targeting the Q_i sites and with antiprotozoal activity have been described more recently, for instance HDQ (Vallières *et al.* 2012), ELQ271 (J Stone Doggett *et al.* 2012), ELQ300 (A. Nilsen *et al.* 2013), or GSK932121 and GW844520 (Capper *et al.* 2015). Yeast is highly sensitive to antimycin and HQNO, HDQ and ELQ271. Point mutations in the Q_i domain modified the inhibitor sensitivity. K228M confers resistance to antimycin and to HDQ; F225L, resistance to HQNO; M221Q resistance to HDQ, ELQ271 and HQNO (Di Rago and Colson 1988), (J Stone Doggett *et al.* 2012), (Vallières *et al.* 2012), ((Gao *et al.* 2003) and references within).

Interestingly, K228 and F225 are replaced by a leucine in the cytochrome *b* of *Plasmodium falciparum* and other apicomplexans, and M221 by a phenylalanine.

In order to study the specificity of the parasite bc_1 complex, we generate a strain that combines the three mutations K228L-F225L-M221F and a fourth change V233F in the vicinity of these mutations. The mutant despite an active bc_1 complex was unable to grow in respiratory medium. Suppressor mutations that restored the growth competence were found.

2 Materials and Methods

2.1 Materials and growth media

Equine cytochrome *c*, decylubiquinone, superoxide dismutase, catalase, ascorbate, TMPD (N,N,N',N'-Tetramethyl-p-phenylenediamine) and CCCP (Carbonyl cyanide 3-chlorophenylhydrazone) were obtained from Sigma Aldrich. The following media were used for the growth of yeast: YPD (1% yeast extract, 2% peptone and 3% glucose), YPGal (1% yeast extract, 2% peptone, 0.1% glucose and 2% galactose), YPeth (1% yeast extract, 2% peptone and 3% ethanol) and YPG (1% yeast extract, 2% peptone and 2% glycerol).

2.2 Yeast strains and plasmids

The mutations were introduced in yeast cytochrome *b* by side-directed mutagenesis and mitochondrial transformation (Hill *et al.* 2003) (Nicholas Fisher, Castleden, *et al.* 2004). In all experiments, control and mutants have identical nuclear and mitochondrial genomes with the exception of the mutations introduced in the cytochrome *b* gene.

2.3 Measurement of decylubiquinol-cytochrome *c* reductase activity

Yeast mitochondria were prepared as in (Lemaire and Dujardin 2008). Briefly, yeast grown in YPGal medium were harvested at mid-log phase. Protoplasts were obtained by enzymatic digestion of the cell wall using zymolyase in an osmotic protection buffer. Mitochondria were then prepared by differential centrifugation following osmotic shock of the protoplasts. Mitochondrial samples were aliquoted and stored at -80 °C. Concentration of bc_1 complex and cytochrome *c* oxidase in the mitochondrial samples was determined from dithionite-reduced optical spectra, using ε=28.5 mM^{-1} cm^{-1} at 562 nm *minus* 575 nm for the haem *b* and ε=21 mM^{-1} cm^{-1} at 603 nm *minus* 625 nm for haem aa_3.

Decylubiquinol-cytochrome *c* reductase activities were determined at room temperature by measuring the reduction of cytochrome *c* (final concentration of 20 μM) at 550 nm *versus* 540 nm over 1-min time-course in 10 mM potassium phosphate pH 7, 0.01% (w/v) lauryl-maltoside and 1 mM KCN. Mitochondria were added to obtain a final concentration of 5-30 nM bc_1 complex. Activity was initiated by the addition of decylubiquinol, a synthetic analog of ubiquinol (final concentrations of 2.5-60 μM). Initial rates were measured as a function of the decylubiquinol concentration. Each measurement was repeated three to five times and the values obtained were averaged. k_{cat} were determined as maximal cytochrome *c* reduction rate per bc_1 complex. Apparent K_M values were estimated from the plots of cytochrome *c* reduction rates *vs* decylubiquinol concentrations, as the decylubiquinol concentrations required to obtain 50% of the observed maximum rate of cytochrome *c* reduction. The mid-point inhibition concentrations (IC_{50}) were determined by inhibitor titration.

In order to assess the production of superoxide, cytochrome *c* reduction rates were recorded in absence and in presence of SOD and catalase, both at 225 units/mL, in potassium phosphate buffer 50 mM pH 7 with 10 μM KCN. Each measurement was repeated at least three times

2.4. Measurement of cytochrome *c* oxidase activity

Cytochrome *c* oxidase activity was measured as oxygen consumption rate using an oxygen electrode at 25°C. Reaction was initiated by the addition of mitochondria in 1 mL of 10 mM potassium phosphate pH 7, 0.01% (w/v) lauryl-maltoside, 5 mM KCL, 2 mM ascorbate, 40 □M TMPD and 40 □M cytochrome *c*. Mitochondria were added to obtain a final concentration of 1-2.5 nM cytochrome *c* oxidase. The measurements were repeated three times and the values averaged.

2.5. Measurement of oxygen consumption by intact cells

Yeast were cultivated in YPGal medium at 28°C in flasks with vigorous agitation for a good aeration. Cells were harvested before the stationary phase (OD 600 nm of 15-18). After centrifugation, the cells were resuspended in fresh YPGal to an OD600 nm of around 250 and immediately used for the assay. Oxygen consumption was monitored in YPGal using an oxygen electrode at 25°C before and after the addition of 15 □M CCCP. The measurements were repeated at least three times and the values averaged. The oxygen consumption activities were determined as oxygen uptake rates per OD_{600nm} cells.

2.6. Aconitase and fumarase measurement using cell extracts

The aconitase and fumarase activities were determined spectrophotometrically by monitoring the formation of cis-aconitate and fumarate at 240 nm and 25°C. Briefly, cell extracts were prepared from 2.0×10^8 cells (OD_{600} ~20) grown on YPGal. Lysis was performed at 4°C in 10 mM MES buffer, pH6 containing 0.6 mM $MnCl_2$ and deprived of oxygen (by bubbling with nitrogen gas) with 0.5 mm glass beads (v/v), by vortexing for 30 s followed by incubation on ice for 30 s, repeating the process seven times. Cell debris was removed by centrifugation at 13,000 rpm for 5 min, and the resulting supernatant was aliquoted and frozen immediately in liquid nitrogen and kept at -80°C. Samples were thawed just before the assay. The aconitase assay mixture contained 50 mM potassium phosphate buffer, pH 7.4, 30

mM sodium isocitrate, 0.6 mM $MnCl_2$. The fumarase assay mixture contained 50 mM potassium phosphate buffer, pH 7.4, 50 mM L-malic acid. Cell extract were added to obtain a final concentration of 150-250 µg of protein. The absorbance changes were measured for 20 min, and the activity was calculated from the slope of the linear portion; ε_{240} = 3.6 mM^{-1} cm^{-1} for cis-aconitate and ε_{240} = 2.44 mM^{-1} cm^{-1} for fumarate.

Results & Discussion

1. Respiratory growth defect but high bc_1 complex activity

We constructed a yeast mutant, named MFKV, combining four cytochrome *b* mutations that replaced yeast residues by the *P. falciparum* equivalents, namely M221F, F225L, K228L and V233F. The mutated residues are located in the Q_i domain

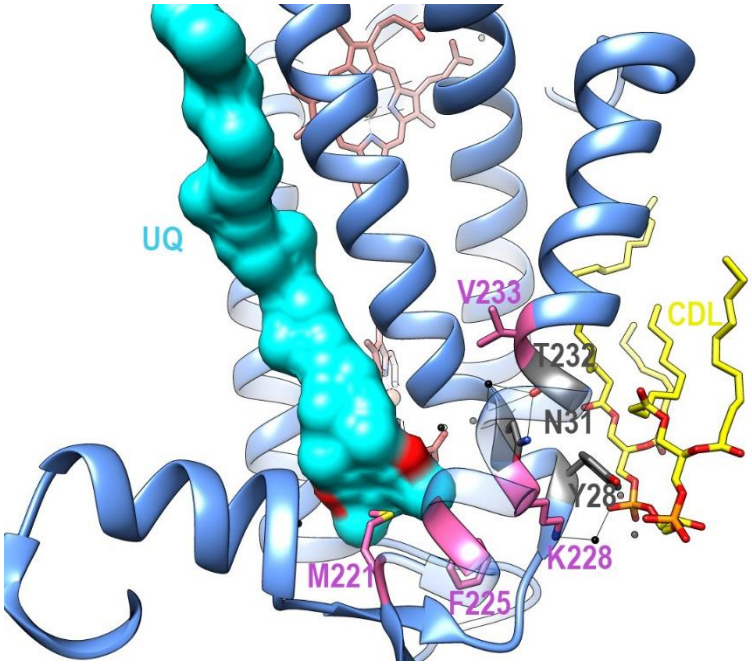

Figure 1. Residues of Q_i site M221, F225, K228, V233 (pink); also Y28, N31 F227 and T232 (gray). The cytochrome *b* (ruban) is in blue; ubiquinone-6 (UQ) (surface) is in cyan; the two oxygens are colored in red. The Cardiolipin is in yellow. Water molecules are presented as black dots.K228 and cardiolipine can both form a hydrogen bond with a water molecule. PDB code of the structure used is 1KB9.

The mutant displayed a severe respiratory medium deficiency (Fig.2). A slight growth could be seen after a long incubation. However, when we monitored the catalytic activity of the bc_1 complex, measured as quinol cytochrome *c* reduction using purified mitochondria, a wt rate was observed (Table 1). We then checked whether the apparent high rate of cytochrome *c* reduction might be due to SO over-production by the bc_1 complex during the assay. We thus monitored SO production as the superoxide dismutase (SOD)-sensitive rate of cytochrome *c* reduction (Materials and Methods). The difference between the reduction rate in the absence and the presence of added SOD gives the contribution of the cytochrome *c* reduction by SO to the overall cytochrome *c* reductase activity. No SO production was detected (not shown), in contrast to the observed SO overproduction caused by *Plasmodium*-like mutations in the Q_o site (Song, Clain, Iorga, Vallières, *et al.* 2015). Thus in the conditions of the assays, there was no indication of a major Q-cycle dysfunction in the MFKV mutated bc_1 complex.

However the first reaction of quinol oxidation in the Q_o site being the rate limiting step of the overall reaction ((Crofts *et al.* 2013) and references within), Q_i site mutations might still impair and slow down the rate of quinone reduction without affecting the rate of cytochrome *c* reduction.

As a control, the cytochrome oxidase activities of the wt and mutant MFKV were monitored using mitochondrial preparations (Materials and Methods). The rates measured were 808 +/- 61 s^{-1} and 942

+/- 137 s^{-1} for the wt and the mutant, respectively. Thus, as expected, the activity of cytochrome oxidase was unaffected in the mutant.

We then analysed the effect of double change F225L, K228L (FK) and single mutations on the respiratory growth competence and bc_1 complex activities (Table 1 and Fig.2). The single mutations had no impact on respiratory growth and moderate or no effect on the complex activity. By contrast, the double mutation resulted in a complete respiratory growth deficiency while the activity of the bc_1 complex displayed a two-fold decrease. No SO production was detected in the bc_1 activity assay (determined as above, not shown). The double mutation (FK) appeared to have a more severe effect than the combined four mutations (MFKV): no respiratory growth was detected even after long incubation and the bc_1 complex was decreased from 50 s^{-1} for MFKV to 28 s^{-1} for FK. Thus the combined replacement of F225 and K228 by a leucine blocks the respiratory growth. The addition of M221F and V233F had a slight correcting effect. FK and MFKV, and the single mutant K228L showed a higher kM for quinol, suggesting that a slight modification in binding environment or the accessibility of the quinole/quinone into the site.

Table 1. bc_1 complex activity and respiratory growth

strain	mutations	k_{cat} (s^{-1})	K_M (□ M)	Respiratory growth
WT		53 +/- 1.4	3.1 +/-	+++
	combined mutations			
MFKV	M221F, F225L, K228L, V233F	48 +/- 2.2	7.0 +/- 0.8	-+
FK	F225L, K228L	28 +/- 1.3	8.0 +/- 0.7	-
	single mutations			
M221F	M221F	70 +/- 4.3	4 +/- 0.3	+++
F225L	F225L	50 +/- 1.9	3.0 +/- 0.1	+++
K228L	K228L	38 +/- 0.5	6.9 +/- 0.3	+++
	Suppressors issued from MFKV			
SUP1	M221F, F225L, K228L, **T232I**, V233F	50 +/- 6.1	3.4 +/- 0.4	
SUP2	**N31D**, M221F, F225L, K228L, V233F	27 +/- 2.9	4.3 +/- 0.2	
SUP3	**Y28S**, M221F, F225L, K228L, V233F	43 +/- 1.3	4.1 +/- 0.1	
SUP5	M221F, F225L, **K228M,** V233F	40 +/- 1.6	4.5 +/- 0.6	
SUP6	M221F, **F225** , K228L, V233F	55 +/- 3.6	3.0 +/- 0.3	

bc_1 complex activities were monitored as described in Materials and Methods. k_{cat} were determined as the maximum cytochrome *c* reduction rate per bc_1 complex. Apparent K_M values were estimated from the plots of cytochrome *c* reduction rates vs quinol concentrations, as the quinol concentrations required to obtain 50% of the observed maximum rate of cytochrome *c* reduction. The suppressor mutations are in bold. Respiratory growth was recorded after few day incubation on YPG plate. +++, growth after three day culture; -+, very weak growth after six day culture; -, no growth after > six day culture.

2. Suppressor mutations in cytochrome *b*

Using mutant MFKV, we selected clones with restored respiratory growth. To that end, several subclones of the mutant were grown in YPD, and then incubated on respiratory medium (YPG). Respiratory competent clones appeared after two to four week incubation. Eight independent clones (each from different subclones) displaying a vigorous growth were then analysed. The suppressor mutations were identified by sequencing the cytochrome *b* gene. The secondary mutation T232I was found in four clones; the other suppressor mutations, namely N31D, Y28S, K228M and the reversion back to wt residue F225, were observed once. Interestingly, the suppressor mutations mapped in the Q_i domain, could be part of a proton pathway to the Q_i site (Fig.1).

The respiratory growth competence and bc_1 complex activities of the suppressors were assessed (Table 1 and Fig.2). The secondary mutations restored a good growth. The bc_1 complex activities varied from a twofold decreased activity as compared to wt (27 s^{-1} SUP2) to wt level (SUP1 and SUP6). The K_M for quinol was decreased (3 to 4.5 □M) and thus closer or identical to wt 3.1 □M, suggesting that the suppressor mutations restored a correct quinol binding or accessibility. None of the suppressors showed SO overproduction in the bc_1 assay (not shown). It could be noted that while FK and SUP2 displayed the same twofold decreased bc_1 complex activity as compared to wt, FK was unable to grow on respiratory medium and SUP2 showed a good growth competence.

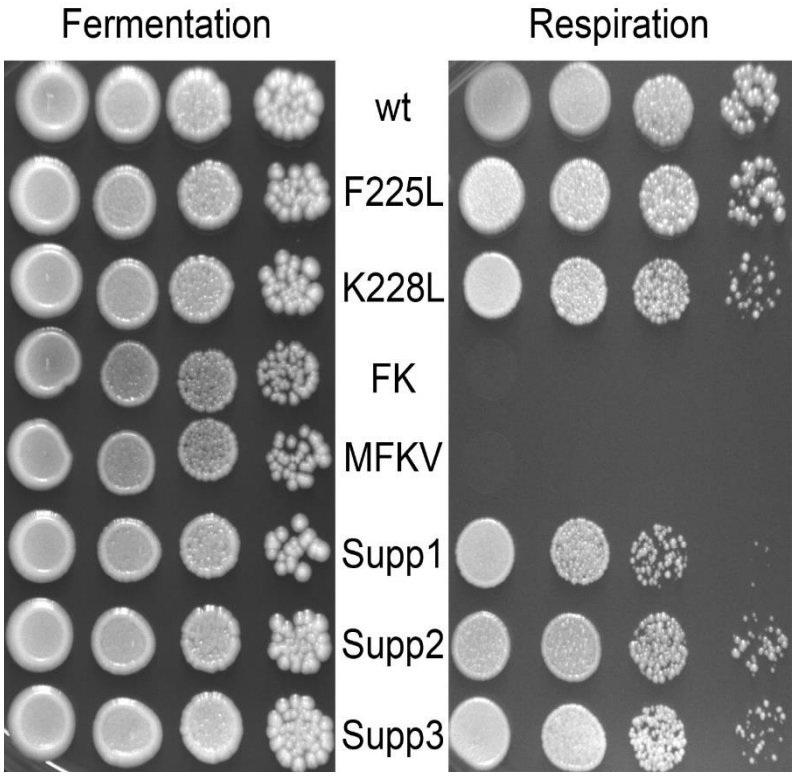

Figure 2. The respiratory competence of mutants compared to wild-type. Serial dilutions in water of cells pregrown on glucose plates were spotted onto plates containing either glucose (YPD, fermentative medium) (left) or glycerol (YPG, respiratory medium) (right) and incubated for 3 days at 28°C. In fermentation where glucose is provided, cells don't need a functional respiratory chain to grow, and all the mutants grow at similar rate. In respiration where only glycerol is provided, cells need a functional respiratory chain to metabolize glycerol to grow, the rate of growth depends on the activity of the respiratory chain.

3. Effect of CCCP on cells oxygen consumption

We monitored the oxygen consumption activity of wt and mutant cells (Materials and Methods). Oxygen consumption rate (mM O_2 per min per OD cells) was measured as described in Materials and Methods. The measurements of wt, MFKV and FK have been repeated seven times and with cells from three different cultures.

Table 2: oxygen consumption rate of wt and mutant cells

strain	oxygen consumption rate (mM O_2 ·min^{-1} ·OD^{-1})	
	without cccp	with cccp
wt	6.9 +/-1.3	20.9 +/- 3.2
MFKV (M221F, F225L, K228L, V233F)	2.1 +/- 0.8	23.3 +/- 3.2
FK (F225L, K228L)	0.5 +/-0.2	5.9 +/- 1.6
F225L	5.3 +/- 0.2	19.0 +/- 1.8
K228L	5.6 +/- 0.2	24.1 +/- 0.6

As presented in Table 2, MFKV and FK mutants displayed low oxygen consumption activities in physiological conditions (without added uncoupler): 30% and 7% of the wt rate, respectively. This clearly reflects the respiratory growth deficiency. Interestingly, upon cccp addition, a 10-fold increase in oxygen consumption rate was observed in the mutant cells, compared to the expected 3-fold increase observed with wt cells. Thus in presence of cccp, MFKV oxygen consumption reached wt level. FK rate remained lower (28% of wt rate). The single mutation F225L and K228L had much milder effect and their behaviour was closer to that of the wt.

The oxygen consumption activities of the suppressors were also monitored. As showed in Fig. 3, the increase of oxygen consumption rate upon cccp addition was similar to wt. Thus the suppressor mutation restored normal oxygen consumption activity and reactivity to cccp, allowing respiratory growth

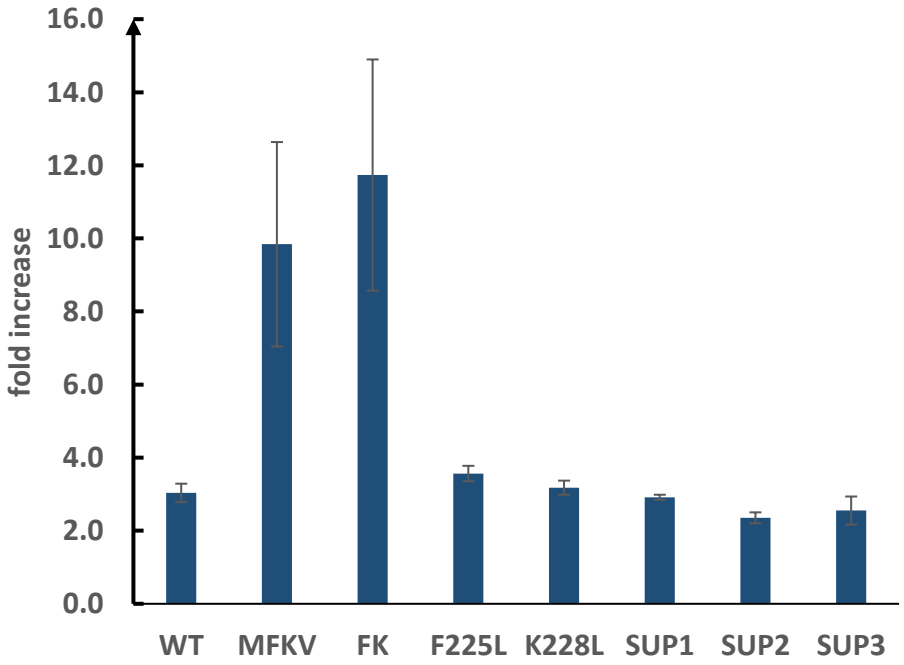

Figure.3 Effect of cccp on oxygen consumption activity. Oxygen consumption activities in absence and presence of cccp were monitored as described in Materials and Methods. The data are presented as the ratio rate in cccp treated cells / rate in untreated cells.

In MFKV, the bc_1 complex activity, and by consequent the respiratory chain, are blocked in physiological condition, *i.e.* in untreated cells with coupled mitochondria. Addition of cccp released that inhibition as the O_2 consumption activity in cccp treated cells reached wt rate.

It should be noted that the MFKV bc_1 complex activities found to reach wt level (Table 1) were assayed in buffer without osmotic protection and with detergent, thus on broken uncoupled mitochondrial membranes

Suppressor mutations restored respiratory growth and normal O_2 consumption activity, presumably by removing the "plug" that hindered the catalytic activity of the complex. As the mutations mapped in a possible proton pathway, the K/cardiolipin pathway (Fig.1), it could be hypothesized that the "plug" that hinders the bc_1 complex activity would be a block in proton entry to the Q_i site.

We could hypothesize that in MFKV, the proton movement from the matrix to Q_i is hindered by the mutations which would slow down the electron transfer and quinone reduction. But, as mentioned above, this is not the limiting step of the overall reaction which explains the wt rate of cytochrome *c* reduction observed in the assays with broken mitochondria (Table 1).

In intact mitochondria, the backpressure of the membrane potential would further slow down the electron flow to Q_i, which results in a severe decrease in bc_1 complex activity, low respiratory function and very poor respiratory growth. Without the backpressure of the membrane potential, the electron movement is facilitated despite the proton hindered movement.

In the suppressors, the proton movement would be restored by the secondary substitutions (in the K/cardiolipine proton pathway) and by consequence, the electron flow would be possible in presence of membrane potential.

FK mutant displayed the same behaviour as MFKV but with a more severe defect.

4. ROS production in MFKV and FK cells

We checked whether ROS were over-produced in MFKV and FK cells as a result of inhibited bc_1 complex. To that end, we measured the activity of aconitase, a tricarboxylic acid (TCA) cycle, located in the mitochondrial matrix and known to be highly sensitive to ROS attack. As a control, the activity of the TCA enzyme fumarate that is not sensitive to oxidative damage was monitored in parallel. As shown in Fig.4, the aconitase activity was over two-fold decreased in the mutants, clearly indicating that the ROS level was increased.

The increased oxidative stress in the mutants could be due to ROS production by the NADH- and succinate-dehydrogenases. Because of the inhibited bc_1 complex, the electron flux is stopped. Electrons are piled on redox cofactors of the enzymes upstream bc_1 complex and might leak to oxygen producing ROS. The bc_1 complex Q_o site of MFKV and FK could also over-produce ROS. In the mutant cells (with intact mitochondria), the Q_i site mutations would prevent the reduction of quinone. The electrons would accumulate on haem b_L, which could result in a higher semiquinone level in the Q_o site and SO overproduction. It has been shown that an increased membrane potential that favoured the backward movement of electrons, or the binding of antimycin in the Q_i site resulted in higher ROS production at the Q_o site (Rottenberg, Covian, and Trumpower 2009) . SO production was not observed in the bc_1 complex assays, which was performed, as mentioned above, on broken uncoupled mitochondria.

The ROS over-production at the Q_o site (and upstream the bc_1 complex) might exacerbate the respiratory growth defect of MFKV and FK.

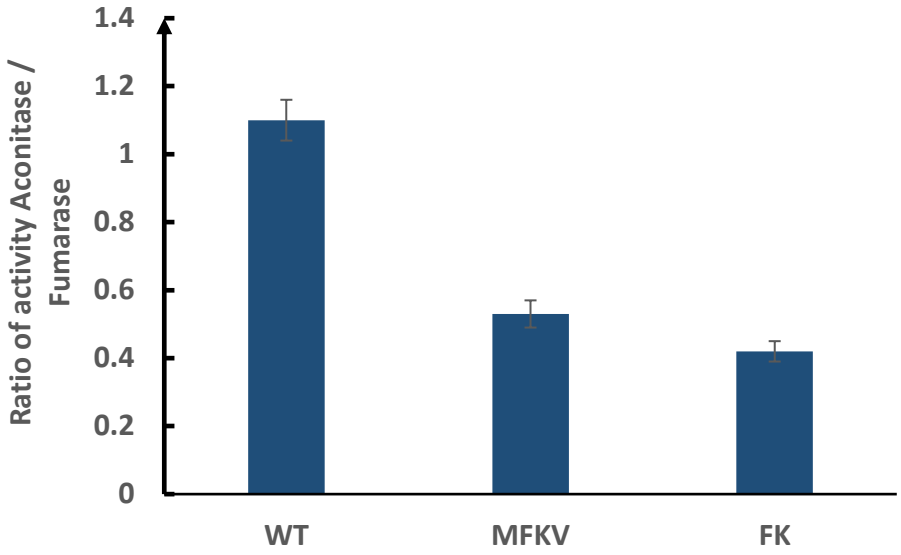

Figure. 4 Activity of the ROS-sensitive aconitase in wt and mutant cells. Aconitase and fumarase activities were assessed spectrophotometrically as described in Materials and Methods. The data are presented as the ratio of the aconitase activity and the fumarase activity. Values represent the averages of four-five independent experiments, with error bars representing standard deviation.

5. *Plasmodium* Q_i site

The introduction of two *Plasmodium*-like mutations (K228L, F225L) in the Q_i domain of yeast bc_1 complex result in a severe respiratory defect by, probably hindering the proton movement to the Q_i site.

K228 are well conserved in the 306 fungi and protozoa cytochrome *b* sequences downloaded from online GenBank database except in *Plasmodium spp.*. The parasite has a leucine at that position. K228, located on helix E, is supposed to form hydrogen bonds with the backbone of Y28 of helix A, moreover, another hydrogen bond with a water molecule that binds to the hydroxyl group of Y28. Whilst in the parasite L228 can't make these connection with S28, so, it is possible that in parasite, the loose interaction between helix A and E is different and results in a different conformation of the Q_i site. Moreover, the water molecule in the parasite equivalent to the K/cardiolipin pathway may be differently positioned since it cannot be stabilized by the K228 and Y28 present in yeast Q_i site. The presence of L228 would be naturally compensated by other changes, so that the proton pathway is functional. Alternatively, other protons channels have evolved in *Plasmodium* Q_i site. It should be noted that in yeast the single mutation, K228L had a minor effect on the bc_1 complex activity.

At position 225, most of the sequences have a phenylalanine or a tyrosine but several species including *Plasmodium spp.* have a different aminoacid (L/I/M). No charged or polar aminoacids have been found at this position. Residue is located inside the Q_i site and in contact with the substrate. It may help the correct positioning of the substrate in the site.

In yeast the combination of K228L and F225L might slightly modify the local structure, altering the accessibility of the protons to the bound quinone. In *Plasmodium*, as mentioned above, the Q_i site wold have evolved to accommodate the residues.

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III. Discussion et perspectives

Le mutant MFKV

Le mutant MFKV a un phénotype particulier. Son complexe bc_1 a une bonne activité quinol -cytochrome *c* réductase, presque identique à l'activité de l'enzyme du contrôle, si ce n'est que la constante d'affinité (K_M) pour le substrat est deux fois plus élevée. Par contre, le mutant ne croît pas sur milieu respiratoire et la consommation d'oxygène des cellules intactes est très faible. Cette consommation augmente de 10 fois après ajout du découplant CCCP (contre une augmentation de trois fois pour le wt).

Nous avons aussi testé la production de SO par le complexe bc_1. Pour ce faire, j'ai comparé l'activité de réduction du cytochrome *c* réductase en présence et en absence de SOD et catalase, en utilisant des mitochondries purifiées (mutant et contrôle). Aucune différence significative n'a été observée, montrant que le complexe bc_1 muté ne produit pas de SO comme nous l'avions observé avec les mutants du site Q_o PF-Y279 et PF-Y279S (Chapitre I-B).

Nous avons ensuite mesuré l'activité aconitase, qui portant un groupe [2Fe-2S] très sensible à l'attaque par les ROS, sur des extraits cellulaires. Une diminution de 50% de l'aconitase a été observée dans le mutant MFKV par rapport au contrôle, ce qui suggère une surproduction de ROS dans les cellules.

Pour essayer d'expliquer le phénotype de ce mutant, nous avons émis l'hypothèse suivante : En conditions physiologiques, dans les cellules, quand les mitochondries sont intactes avec un potentiel de membrane, les mutations MFKV ralentissement significativement l'activité du complexe bc_1. Ceci conduit à une chaîne respiratoire non-fonctionnelle, se traduisant par un défaut de croissance respiratoire et une faible consommation d'oxygène et une surproduction de ROS.

Lorsque les mitochondries ont perdu le potentiel de membrane après ajout de CCCP ou dans les conditions de la mesure d'activité du complexe bc_1, ces mutations ont peu d'effet sur l'activité du complexe bc_1.

Pour aller plus loin dans la compréhension de ce phénotype, nous avons sélectionné des suppresseurs à partir de la souche MFKV qui rétablissent un fonctionnement normal. Les mutations de suppression trouvées sont Y28S/C, N31D, L225F, L228M et T232I. Nous avons aussi étudié des simples mutants et un mutant double portant les deux mutations F225L et K228L, appelé mutant FK. Ce mutant FK a le même phénotype que MFKV. Par contre, les mutations simples n'ont que très peu d'effet.

M221 a une chaîne latérale en contact direct avec l'ubiquinone et est perpendiculaire à son cycle aromatique. Son rôle pourrait être de stabiliser le substrat en orientant correctement son cycle. Une phénylalanine ou une glutamine, avec une chaîne latérale non-ionisable et de longueur similaire pourrait alors remplacer la méthionine dans ce rôle. Le résidu 233 est assez éloigné du cycle aromatique du substrat et des molécules d'eau. La modification V233F n'aura donc pas d'effet majeur sur le fonctionnement du site Q_i. Ceci expliquerait pourquoi nous n'avons pas observé de mutations de suppression à ces deux positions. Par contre, nous avons trouvé des mutations de suppression en position 225 et 228, ainsi aux positions 28, 31 et 232.

Les résidus en position 28, 31, 225, 232 et 228 forment la porte d'entrée de la voie de passage de protons « cardiolipine/K228 ». Les mutations de suppression ont montré qu'en substituant un de ces cinq résidus, l'activité respiratoire et la croissance respiratoire du mutant MFKV peuvent être restaurées.

L'observation de la structure autour de ces résidus montre qu'il y a un réseau de molécules d'eau dans cette zone. Beaucoup de liaisons hydrogène sont probablement présentes et cette zone enrichie en résidus hydrophiles se situe dans une région du cytochrome *b* largement hydrophobe. Cela nous a amené à suggérer que la déficience de croissance respiratoire de MFKV (et FK) - et le rétablissement de la fonction respiratoire dans les suppresseurs - résulterait de modification du transfert de protons vers le substrat. Pour essayer d'expliquer quel pourrait être le rôle du potentiel de la membrane dans l'acheminement des protons de la matrice vers le site Q_i via le passage de « cardiolipine/K228 » (cf. Introduction B.2), nous émettons l'hypothèse qu'avec la porte d'entrée du passage des protons modifiée (mutants MFKV et FK), la réduction de la quinone en quinol dans le site Q_i est rendue plus difficile. En absence de potentiel de membrane, cela n'a pas ou peu d'effet sur l'activité globale du complexe, parce que l'étape limitante du cycle Q est probablement le transfert du premier électron vers le [2Fe-2S] de l'ISP dans le site Q_o. En présence du potentiel de membrane, la situation est différente. Les électrons doivent passer de l'hème b_H à l'hème b_L, puis à la quinone au site Q_i, à « contre-courant » de potentiel de membrane. Le ralentissement du passage des protons vers le site dû aux mutations peut, dans ce cas, résulter dans une diminution plus sévère de l'activité de la réduction de la quinone dans le site Q_i et par conséquent, une diminution de l'activité globale du complexe bc_1. Les mutations de suppression, en modifiant un autre résidu de cette voie de passage de protons, restaureraient le flux d'entrée de protons et un bon fonctionnement du site Q_i, et donc du complexe bc_1.

<u>La voie de passage des protons dans le site Q_i de *Plasmodium*</u>

La combinaison des mutations MFKV / FK dans le site Q_i résultent dans une déficience respiratoire sévère chez la levure, mais ces « mutations » sont naturellement présentes dans le complexe bc_1 de *Plasmodium*. Ce site doit malgré cela être fonctionnel. Il est possible que la position du substrat dans le site Q_i soit légèrement différente chez *Plasmodium* et/ou que ces « mutations » délétères chez la levure soient naturellement compensées par d'autres changements du site Q_i.

Comme nous l'avons mentionné au chapitre II-A, point III., le site Q_i est moins bien conservé que le site Q_o parmi les espèces. Il serait intéressant de faire une analyse comparée des séquences et des modèles de structures, ce qui pourrait donner des informations sur l'évolution du site, et en particulier des voies de passage des protons.

<u>Reconstruction du site Q_i de la levure pour obtenir une version « *Plasmodium* »</u>

Afin d'obtenir un bon outil pour l'étude des inhibiteurs ciblant le site Q_i, nous avons continué à utiliser le site Q_i de levure pour créer un site Q_i *Plasmodium*, en remplaçant une série de résidus par leur équivalent parasitaire. Cette construction génétique est en cours. Ces nouveaux mutants « PF » combineront les mutations des clusters 1, 2 et 3 (Fig.18).

Les mutants du site Q_i version *Plasmodium* ayant un complexe bc_1 actif, s'ils reproduisent bien la sensibilité de l'enzyme du parasite aux inhibiteurs déjà connus ciblant le site Q_i, pourront être utilisés comme outil de criblage pour identifier d'autres inhibiteurs et pour étudier le développement des mutations de résistance.

Cette approche peut aussi être utilisée pour l'étude d'autres pathogènes tels que le *Toxoplasma*, *Pneumocystis* ou des *Oomycetes*. Une reconstruction génétique des sites actifs du complexe bc_1 de ces espèces pourrait être utilisé pour comprendre l'effet des mutations, la sensibilité différentielle et pour le criblage des inhibiteurs spécifiques.

Conclusions générales
&
Perspectives

Conclusions générales & perspectives : les antipaludiques ciblant le complexe bc_1.

L'analyse mutationnelle est une approche classique pour étudier le mécanisme de réaction et la sensibilité différentielle aux inhibiteurs. Utilisant la levure comme organisme modèle, nous pouvons modifier spécifiquement la séquence d'acides aminés du cytochrome *b*, qui forme les deux sites catalytiques et qui est codé par le génome mitochondrial. Nous pouvons donc introduire les mutations de résistance observées chez des organismes pathogènes, comme *Plasmodium*, ou reconstruire les sites pour les rendre plus semblables à ceux du parasite. Cette approche est combinée avec une analyse *in silico* qui permet de mieux comprendre, au niveau structural, l'impact des mutations.

Le site Q_o est le site d'une réaction complexe avec un transfert d'électrons accompagné du transfert de protons. De plus le site est composé de cytochrome *b* et l'ISP. Ces deux sous-unités ont probablement co-évolué pour assurer la bonne structure du site Q_o. Les deux partenaires doivent se réajuster lorsqu'un changement affectant leur interaction apparaît dans l'un d'eux.

Le site Q_o se situe vers l'espace intermembranaire, dans lequel s'accumulent les protons, créant le potentiel membranaire. C'est aussi dans le site Q_o que sont produits les ions superoxide (SO) quand la réaction ne se fait pas correctement. La réaction, dans ce site, est assez bien élucidée mais le mécanisme de production de SO n'est pas tout à fait bien expliqué. Pour mieux le comprendre, il faudrait mieux connaître la (les) localisation(s) du substrat dans le site pendant la réaction.

Beaucoup d'inhibiteurs du complexe bc_1 se lient dans ce site, comme l'antipaludique atovaquone. Nous avons étudié l'effet des substitutions dans le cytochrome *b* sur la sensibilité à cette molécule, chez la levure. Nous avons aussi créé des souches qui miment le site Q_o du *Plasmodium* et de l'Homme. Ces souches, et d'autres mimant le site d'autres pathogènes, pourront être de bons outils pour étudier, voire cribler de nouveaux inhibiteurs du site Q_o d'une certaine espèce et/ou étudier la toxicité potentielle d'un inhibiteur chez une autre espèce, chez l'Homme en particulier.

Chez l'Homme, beaucoup de variations dans le cytochrome *b* ont été recensées et classifiées comme polymorphismes. Mais il se peut que certains polymorphismes aient un impact dans des conditions particulières, par exemple, en présence d'inhibiteurs tels que l'atovaquone, comme nous l'avons montré ici. La données obtenues avec l'outil « levure » donneront des indications sur les risques - accrus ou diminués - de disfonctionnement du complexe bc_1 suite au traitement.

Le site Q_i est moins bien conservé entre les espèces que le site Q_o, ce qui expliquerait une sensibilité différentielle aux inhibiteurs plus prononcée. Pour l'instant, aucun médicament antipaludique ciblant le site Q_i n'est sur le marché. Le site est une bonne cible thérapeutique et des molécules prometteuses sont étudiées.

L'ELQ-400, par exemple, est un inhibiteur prometteur. Cette molécule se lierait dans les deux sites Q_o et Q_i. Il devrait donc être beaucoup plus difficile pour les parasites de développer une forte résistance à cet inhibiteur car cela nécessiterait deux mutations apparaissant simultanément. De plus, nous avons montré qu'une mutation de résistance à l'atovaquone (Y279S/C/A) peut induire une forte diminution d'activité. Deux mutations dans les sites actifs pourraient affecter sévèrement l'activité du complexe.

Le développement de médicaments antipaludiques ciblant de complexe bc_1 requière que les enzymes du parasite et de l'Homme présentent une sensibilité différentielle. C'est le cas pour l'atovaquone et les ELQs,

et est expliquée par des différences structurales dans les sites actifs du complexe. Le développement de ces nouveaux inhibiteurs, ainsi que la prédiction du risque d'apparition de mutations de résistance requière une bonne connaissance de structure et fonction du complexe.

Comme nous l'avons mentionné plus haut, certains polymorphismes pourraient rendre l'enzyme plus ou moins sensible aux inhibiteurs. La variation génétique des patients et des pathogènes devrait être prise en considération. Une connaissance approfondie de l'interaction moléculaire entre inhibiteurs et site de liaison est donc importante.

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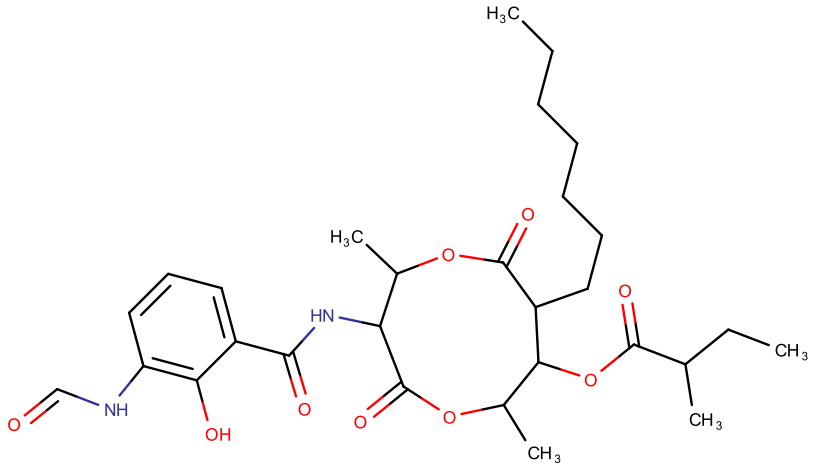
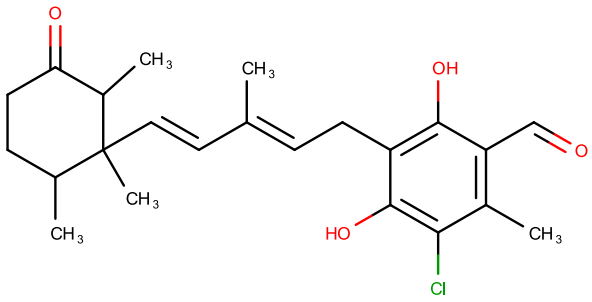
Annexe : les structures chimiques des molécules

par ordre alphabétique

Antimycine



Ascochlorine



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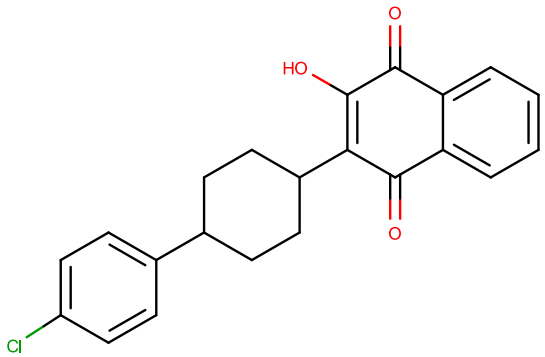
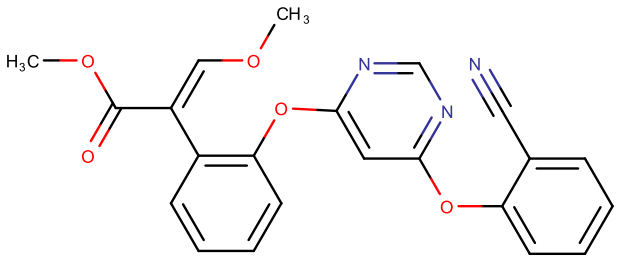
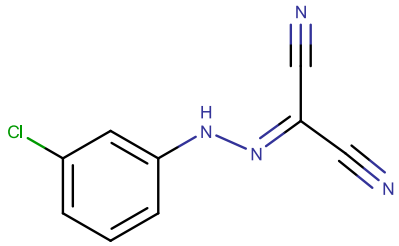


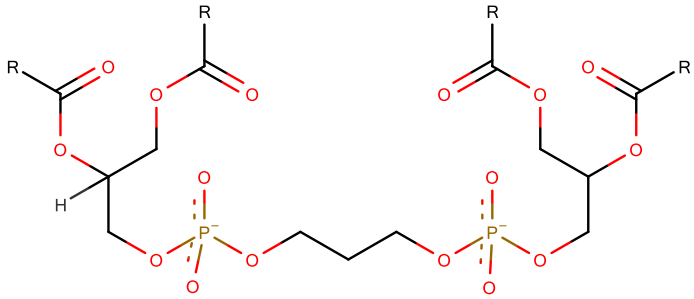
Azoxystrobine



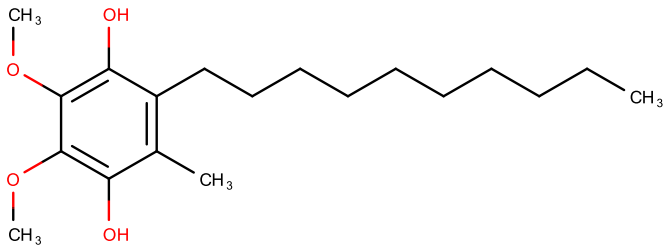
CCCP (Carbonyl cyanide m-chlorophenyl hydrazone)



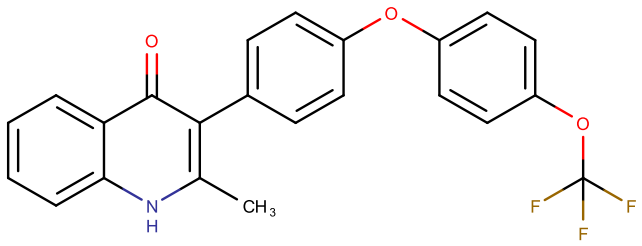
CDL (Cardiolipine)



Décylubiquinol



ELQ-271



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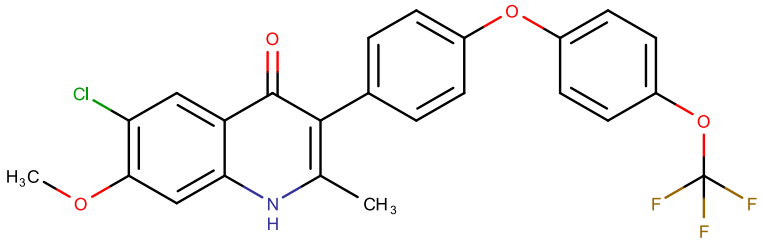
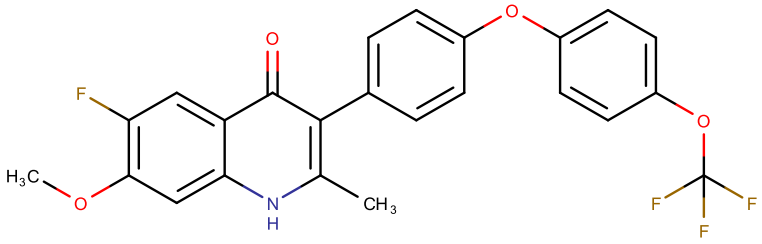
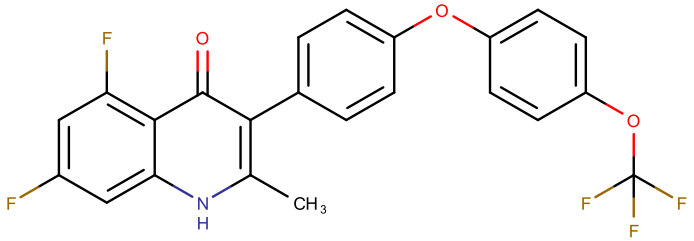


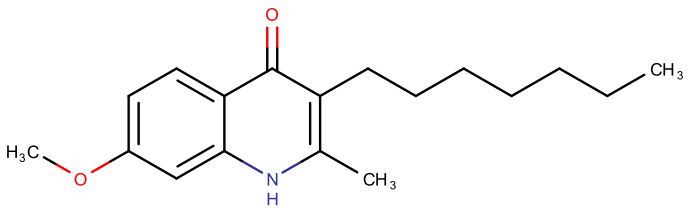
ELQ-316



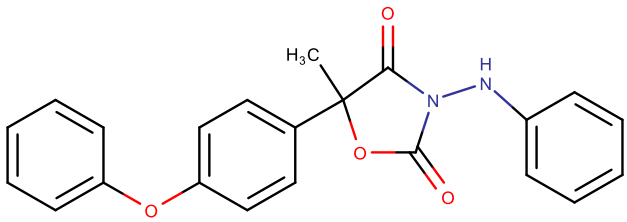
ELQ-400



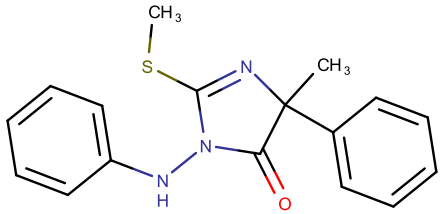
Endochine



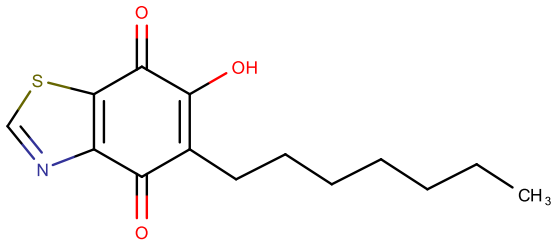
Famoxadone



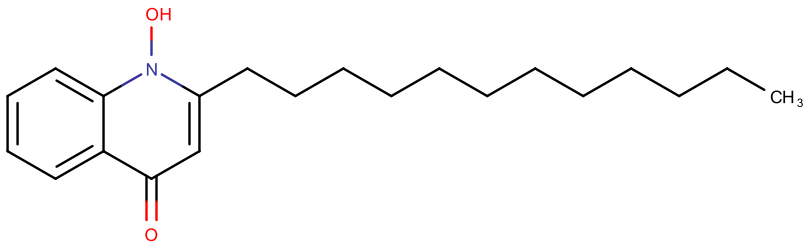
Fenamidone



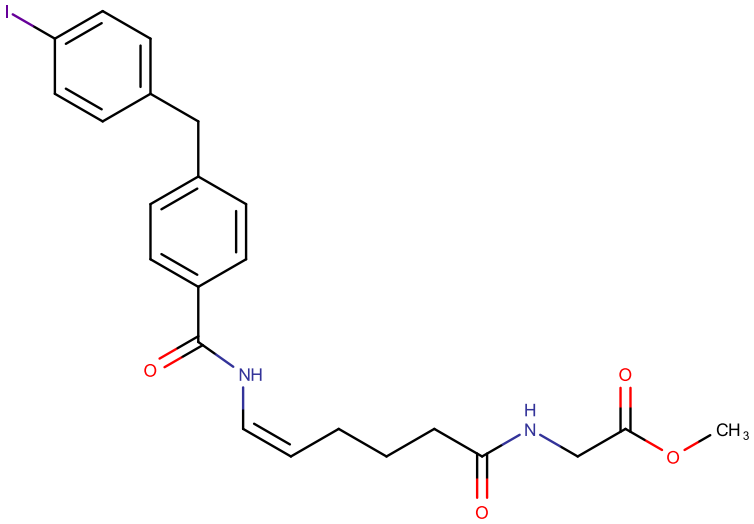
HDBT (5-HEPTYL-6-HYDROXY-1,3-BENZOTHIAZOLE-4,7-DIONE)



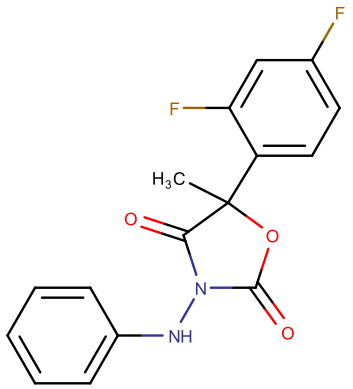
HDQ (1-Hydroxy-2-Dodecyl-4(1H)Quinolone)



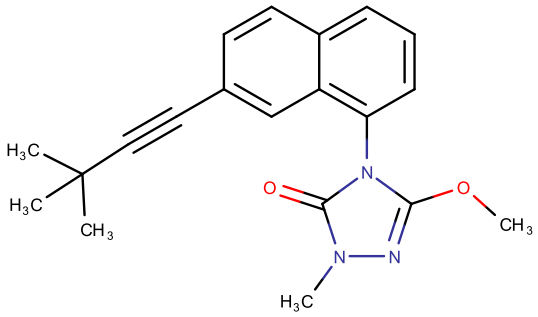
Iodinated analogue of the polyketide Crocacine-D



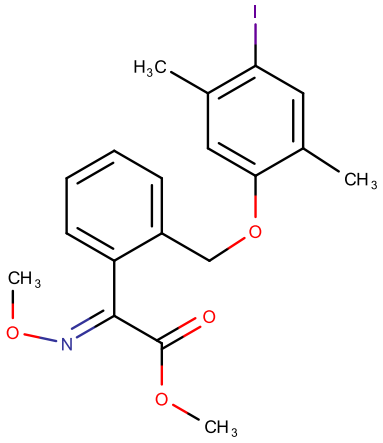
Jg144 ((5S)-5-(2,4-difluorophenyl)-5-methyl-3-(phenylamino)-1,3-oxazolidine-2,4-dione)



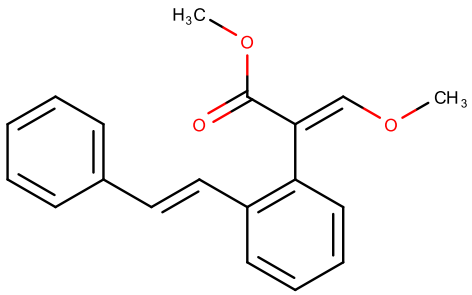
JZZ (triazolone)



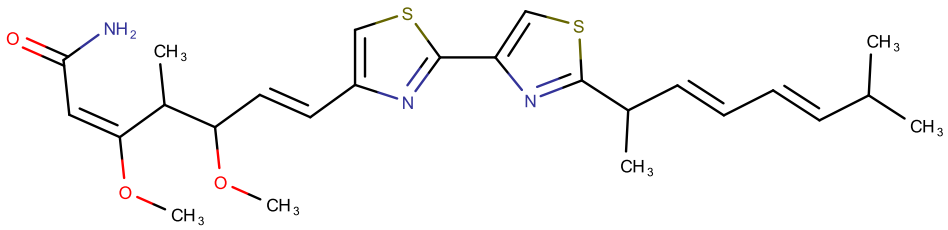
Kresoxym-I-dimethyl



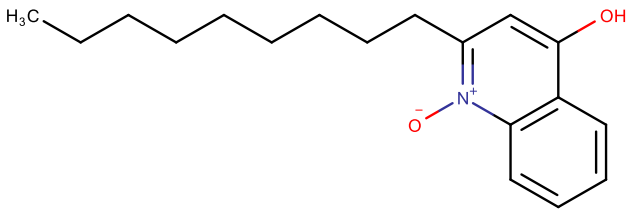
MOAS (Methoxy Acrylate Stilbène)



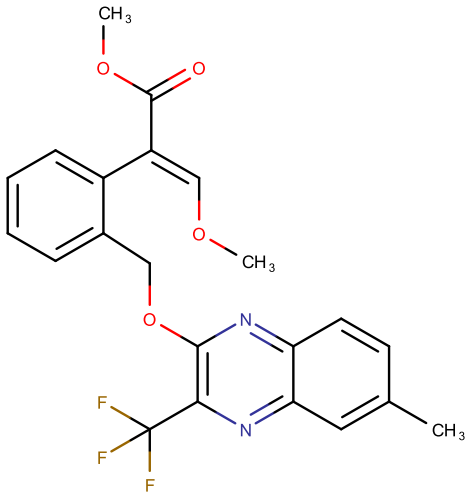
Myxothiazole



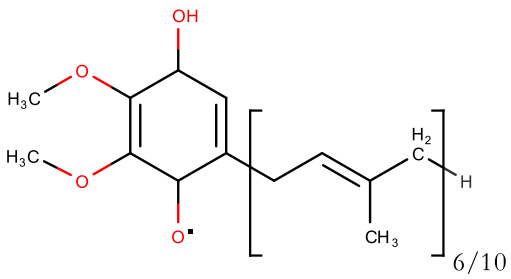
NQNO



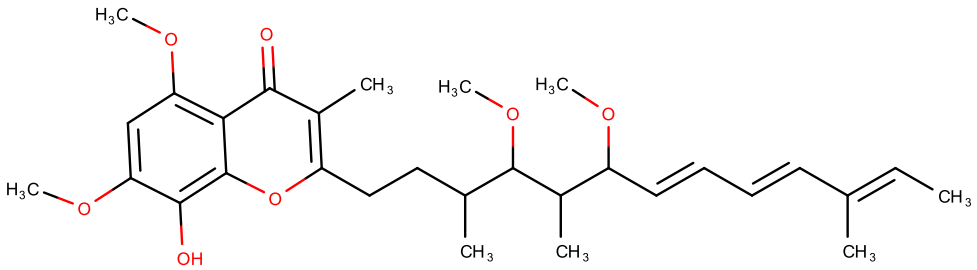
Pfvs-designed MOA



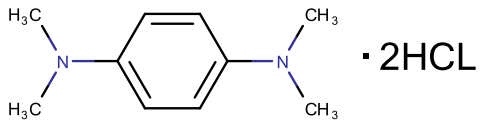
Semiquinone-6/10



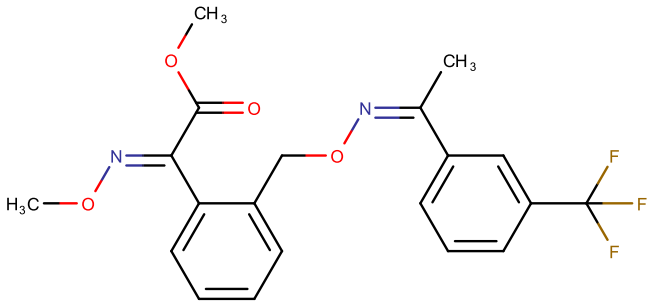
Stigmatelline



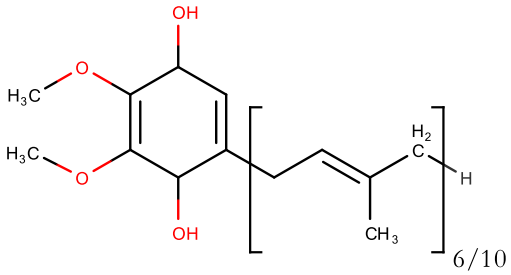
TMPD (N,N,N',N'-Tetramethyl-p-Phenylenediamine)



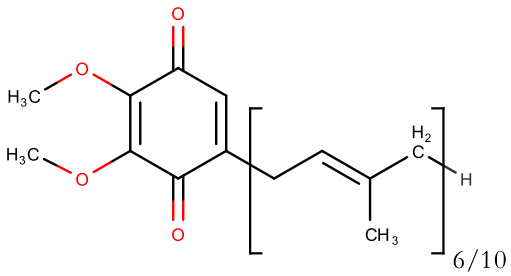
Trifloxystrobine



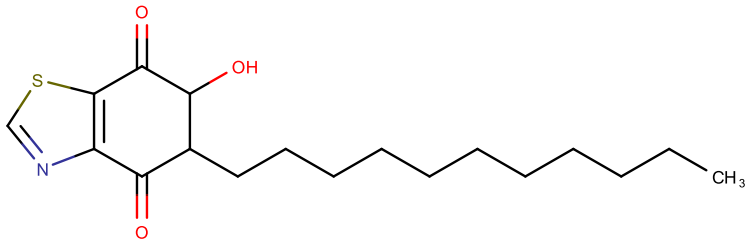
Ubiquinol-6/10



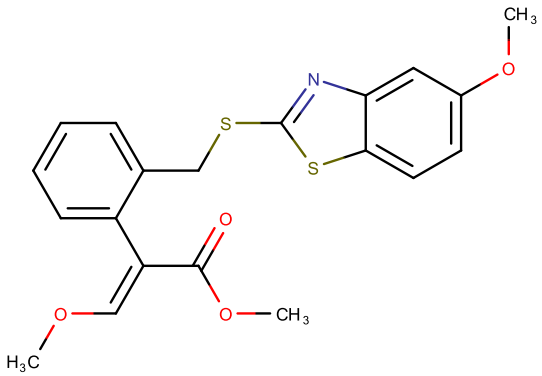
Ubiquinone-6/10



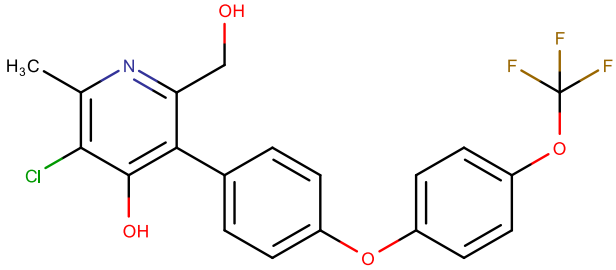
UHDBT (6-HYDROXY-5-UNDECYL-1,3-BENZOTHIAZOLE-4,7-DIONE)



Y52



4(H)-pyridone GSK932121



4(H)-pyridone GW844520