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REVIEW ARTICLE

The cytochrome *bc*₁ complex as an antipathogenic target

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The cytochrome *bc*₁ complex is a key component of the mitochondrial respiratory chains of many eukaryotic microorganisms that are pathogenic for plants or humans, such as fungi responsible for crop diseases and *Plasmodium falciparum*, which causes human malaria. Cytochrome *bc*₁ is an enzyme that contains two (ubi)quinone/quinol-binding sites, which can be exploited for the development of fungicidal and chemotherapeutic agents. Here, we review recent progress in determination of the structure and mechanism of action of cytochrome *bc*₁, and the associated development of antimicrobial agents (and associated resistance mechanisms) targeting its activity.

Keywords: cytochrome *bc*₁; electron transport; fungicide; G143A; malaria; QoI/QiI; resistance

Introduction and general enzymatic mechanism of the cytochrome *bc*₁ complex

The cytochrome *bc*₁ complex (respiratory complex III, Cyt *bc*₁, EC: 1.10.2.2) of eukaryotic mitochondrial and prokaryotic energy-transducing membranes is a proven target for antimicrobial agents of medical and agricultural interest [1–7]. This enzyme, which is widespread in nature, functions as a protonmotive ubiquinol:cytochrome *c* oxidoreductase, conserving and converting the Gibbs energy obtained from the exergonic oxidation of ubiquinol by cytochrome *c* into a transmembrane proton gradient which may be harnessed for other endergonic processes, typically ATP synthesis by an F_oF₁ ATP synthase [8,9].

Cyt *bc*₁ is a multimeric, homodimeric complex. In eukaryotes (the focus of this review), the monomer consists of 10–11 discrete polypeptides, with a

molecular mass of approximately 240 kDa [8,10]. All subunits are nuclearly encoded with the exception of cytochrome *b* (cyt *b*), which is encoded by the mitochondrial genome. Three subunits – cyt *b*, the Rieske [2Fe2S] iron–sulfur protein (ISP) and cytochrome *c*₁ (cyt *c*₁) – form the highly conserved electron- and proton-transferring catalytic engine of the enzyme (Fig. 1), embedded within the inner mitochondrial membrane (IMM). The [2Fe2S] cluster and haem group of cyt *c*₁ form the ‘high potential’ chain of cyt *bc*₁, with midpoint redox potentials ($E_{m,7}$) of approximately +280 and +240 mV, respectively. The cytochrome *b* polypeptide forms the ‘low potential’ transmembrane electron transfer pathway and binds two b-type haems, designated *b*_L and *b*_H, with $E_{m,7}$ values of approximately –100 and +50 mV, respectively. (For reference, the $E_{m,7}$ for the 2 electron redox

Abbreviations

Δp, protonmotive force; AOX, (mitochondrial) alternative oxidase; cyt, cytochrome; DHODH, dihydroorotate dehydrogenase; $E_{m,7}$, (redox) midpoint potential at pH 7; ED, extrinsic domain; EPR, electron paramagnetic resonance; FRAC, Fungicide Resistance Action Committee; HHDBT, *n*-heptyl-6-hydroxy-4,7-dioxobenzothiazole; IMM, inner mitochondrial membrane; IMS, (mitochondrial) intermembrane space; ISP, (Rieske) iron–sulfur protein; NQNO, *n*-nonyl-quinoline N-oxide; MDR, multiple drug resistance; MOA, methoxyacrylate; mtDNA, mitochondrial DNA; Q_i, site of (ubi)quinone reduction within cytochrome *b*; QiI, Q_i-site inhibitor; Q_o, site of (ubi)quinol oxidation within cytochrome *b*; QoI, Q_o-site inhibitor; ROS, reactive oxygen species; SAR, structure–activity relationship; SHAM, salicylhydroxamic acid; SQ_i, semiquinone bound at the Q_i site of cytochrome *b*; SQ_o, semiquinone bound at the Q_o site of cytochrome *b*; UHDBT, *n*-undecyl hydroxy dibenzothiazole.

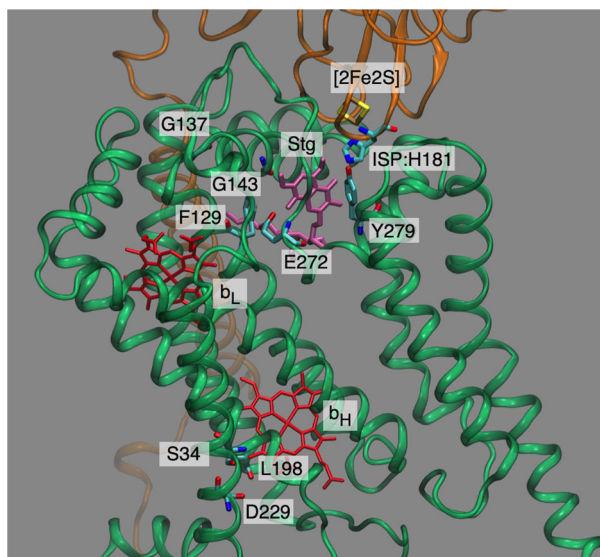


Fig. 1. Cartoon representation of the atomic structure of cyt *b* (green) and the ISP (orange) of yeast cyt *bc*₁ (1EZV.PDB [39]) with stigmatellin (Stg, pink) bound at Q_o. The ISP [2Fe2S] cluster, cyt *b* haems (red) and side chains of interest as discussed in the text are represented in wireframe form.

chemistry of the ubiquinone/ubiquinol redox couple is approximately +60 mV.) During the catalytic cycle, which is described in more detail below, cyt *bc*₁ oxidises two molecules of ubiquinol in a stepwise manner, reducing two molecules of the soluble acceptor cytochrome *c* and one molecule of ubiquinone. The protonmotive activity inherent to this catalytic cycle results in the deposition of four protons against the electrochemical gradient into the intermembrane space, per two electrons transferred to cyt *c*, with the concomitant uptake of two protons from the mitochondrial matrix [9,11,12].

The ubiquinol- and ubiquinone-binding sites of cyt *bc*₁ are termed Q_o and Q_i, for the sites of (ubi)quinol oxidation and (ubi)quinone reduction, respectively. They form exploitable targets for competitive inhibitors and are located within cyt *b*, disposed on opposite sides of the IMM and linked by haems *b*_L and *b*_H. The bifurcating electron transfer chemistry between the high and low potential chains of cyt *bc*₁ is highly unusual and best understood through the framework of the Q-cycle model (Fig. 2), originally proposed by Peter Mitchell in 1975 and substantially developed by others since [13–18]. The basic tenets of the model are this. A ubiquinol molecule binds at the quinol oxidation (Q_o) site of cyt *b*, located towards the intermembrane space (i.e. electrochemically positive) side of the IMM. This ubiquinol serves as a one-electron

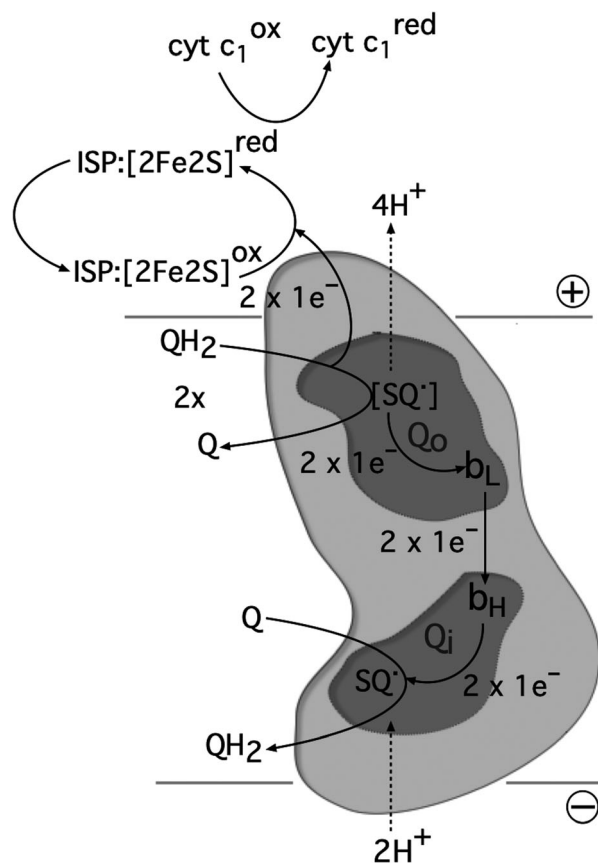


Fig. 2. Schematic sketch of the electron and proton transfer pathways of the cyt *bc*₁ Q cycle. Cyt *b* is represented in light grey, with the Q_o/Q_i pockets in dark grey. 'Red' and 'ox' refer to the reduced and oxidised states of the various redox carriers. The positive and negative sides of the energised inner mitochondrial membrane are indicated accordingly.

reductant for the [2Fe2S] cluster of the mobile extrinsic domain of the ISP (the ISP-ED), the entry point for the high potential chain within *bc*₁. The ISP-ED effectively acts as a tethered substrate and kinetic gate facilitating electron bifurcation, although it is important to note that this in itself is not the only factor controlling the bifurcation reaction [19–22]. This electron transfer reaction from ubiquinol to the ISP-ED (which is rate-limiting for the chemistry at Q_o) generates a strongly reducing semiquinone (SQ_o) radical at Q_o. The reactivity of SQ_o must be carefully controlled to minimise energetically wasteful (and potentially harmful) side reactions with molecular oxygen. The mechanisms by which this radical intermediate – an elusive species – is managed at Q_o are subject to much investigation, discussion of which is beyond the scope of this review, but the interested reader is referred to [23–26] for further details. Protons released from

ubiquinol oxidation at Q_o are deposited into the intermembrane space (IMS) bulk phase. SQ_o is of sufficiently negative redox potential (c. −160 mV) to reduce the low potential chain within cyt *b*, with electron entry at haem *b*_L, which is then rapidly oxidised by haem *b*_H, the electron donor to Q_i-bound ubiquinone. This one-electron transfer reaction creates a tightly bound (and relatively stable) semiquinone species at Q_i (SQ_i), which is reduced to ubiquinol by a second turnover of the Q_o site and uptake of two protons from the mitochondrial matrix, and the cycle is complete. The tight binding of SQ_i at Q_i ensures that the thermodynamics are favourable for the first one-electron transfer reaction to substrate ubiquinone at this site, which otherwise would be energetically uphill [9].

Structure, function and inhibition of the cyt *bc*₁ Q_o and Q_i sites

High-resolution (< 3.0 Å) atomic structures of cyt *bc*₁ cocrystallised with a variety of Q_o/Q_i-site occupants are available for the chicken, bovine, yeast (*Saccharomyces cerevisiae*) and bacterial (*Rhodobacter sphaeroides*, *R. capsulatus* and *Paracoccus denitrificans*) enzymes (see survey in Ref. [10]). Recently, single-particle cryo-electron microscopy has proven to be useful tool for structural investigation of this enzyme [27]. No crystal structures of cyt *bc*₁ are available from human or plant pathogen sources; however, the yeast enzyme has proved a useful and genetically amenable model in many instances [1,4,28–32].

The Q_o site within cyt *b* is composed from components encompassing the C-terminal domain of transmembrane helix C, surface helix cd1 and the region encompassing the ‘PEWY’ (Pro₂₇₁-Glu-Trp-Tyr₂₇₄, yeast notation) loop/ef helix to transmembrane helix F1 (Fig. 3). It forms a large, bifurcated and predominantly hydrophobic volume [33,34]. Cyt *bc*₁ has been cocrystallised with a variety of competitive inhibitors for the Q_o site (see Ref. [10,34] and references therein, also Ref. [4]), but despite extensive efforts, there are no atomic structures available for the enzyme with substrate ubiquinol bound at Q_o. Ligands binding within Q_o may be broadly classified as ‘*b*_L-distal’ or ‘*b*_L-proximal’ species depending on their positioning within the site with respect to haem *b*_L. *b*_L-distal inhibitors, such as stigmatellin, *n*-nonyl quinoline *N*-oxide (NQNO), *n*-undecyl hydroxy dibenzothiazole (UHDBT) and atovaquone, bind in a region of Q_o in close proximity to the ISP (Fig. 1), often forming a strong hydrogen bond to a histidine ligand (H181) of the [2Fe2S] cluster, and restricting the movement of

the ISP-ED in crystallographic studies [10,19,34]. As such, this class of inhibitor may also be classified as ‘Pf’ [‘P’ referring to Q_p, an alternative nomenclature for Q_o (i.e. the positive side of the energy coupling membrane), with ‘f’ indicating that the ISP-ED is fixed in position] [34]. These inhibitors may also alter the redox and EPR spectroscopic properties of the [2Fe2S] cluster, a clue to their mode of action prior to the elucidation of the atomic structure of cyt *bc*₁. To complicate matters, it should be noted that not all *b*_L-distal/Pf inhibitors form H-bonding associations with the ISP-ED. Famoxadone (an oxazolidinedione-containing synthetic fungicide) is a useful case in point here, demonstrating direct Q_o ligand/ISP-ED interactions are in themselves insufficient to retard the movement of the ISP-ED, and may arise from ligand-induced perturbation of the protein fold around this region of cyt *b* [35]. *b*_L-distal inhibitors may also form hydrogen-bonding interactions with the carboxylate moiety of E272 in the cyt *b* Q_o ef helix (this residue is in close proximity to haem *b*_L), causing this side chain to rotate away from its inward pointing disposition in the uninhibited enzyme. This is observed with stigmatellin and NQNO, but not UHDBT [34,36].

In contrast to *b*_L-distal inhibitors, *b*_L-proximal inhibitors, such as the natural antibiotics myxothiazol and strobilurin, and synthetic fungicides such as azoxystrobin characteristically have hydrogen-bonding interactions with the backbone amide moiety of cyt *b* residue E272 at Q_o (Fig. 1), with the side chain of this residue oriented away from the aqueous phase. Often such inhibitors possess a methoxyacrylate pharmacophore, or derivative of such [34,37]. Generally, *b*_L-proximal inhibitors do not interact with the ISP-ED or affect its mobility, and so this class of compound is also referred to as ‘Pm’ (with ‘m’ indicating that the ISP-ED is mobile, or, at least, not docked at the IMS-facing surface of the Q_o site within cyt *b*) [34].

The Q_i site, located within cyt *b* on the opposite side of the IMM to Q_o, forms the site of quinone reduction within cyt *bc*₁. Structurally, it is formed from the C-terminal region of surface helix a, the N-terminal region of transmembrane helices A and E and the C-terminal regions of helices D (Fig. 3), and, in contrast to Q_o, contains no contributions from the ISP. Cyt *bc*₁ has been cocrystallised with Q_i-bound substrate ubiquinone in the chicken, bovine and yeast enzyme, where it is observed that the benzoquinone moiety forms hydrogen bonds with Q_i residues H202 and S206 (yeast notation) [38–41]. Q_i-bound quinone is in close proximity (3.5 Å shortest separation) to haem *b*_H, facilitating rapid electron transfer [12,17].

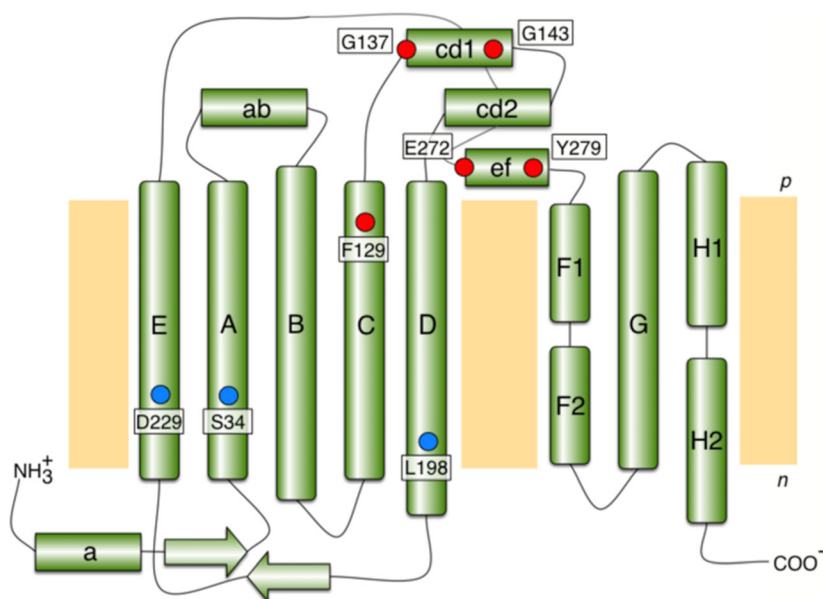


Fig. 3. Cartoon of the secondary structure and membrane disposition of *cyt b*, displaying the location of Q_o (red) and Q_i (blue) residues of interest (*Saccharomyces cerevisiae* notation) as discussed in the text. Transmembrane and surface helices are identified in upper and lower cases, respectively. 'n' and 'p' refer to the negative and positive sides of the energised inner mitochondrial membrane.

*Cyt bc*₁ has been cocrystallised with a variety of Q_i inhibitors (QiIs), such as the natural antibiotics antimycin A and ascochlorin, and synthetic pyridones [5,10,41–43]. These inhibitors tend to be structurally diverse molecules, and may not resemble simple variants of benzoquinones, as is often observed for Q_o inhibitors (QoI). Mechanistically, the redox chemistry catalysed at Q_i is less elaborate than that at Q_o [9,17], and there is no equivalent of the haem proximal/distal classification for QiI as observed with QoI. Notably, Q_i inhibition can lead to increased superoxide production from *cyt bc*₁ due to electron accumulation on the low potential chain (and increased SQ_o occupancy) within this enzyme [44–46], which, due to the resulting oxidative stress, may increase the efficacy of such compounds as antimicrobial agents. It is often overlooked that b_L -proximal QoI (such as myxothiazol) can also stimulate ROS production by *cyt bc*₁, presumably as SQ_o generation can still proceed due to dual-site occupancy at Q_o , allowing one-electron oxidation of substrate quinol by the ISP (but blocking reduction in *cyt b*) [47].

Finally, we note that some compounds, such as the fungal antibiotic ascochlorin, the synthetic fungicide ametocradin and the synthetic antimalarial compound endochin ELQ-400, may act as dual-site Q_o/Q_i inhibitors [31,32,43]. Such molecules are of particular interest as antimicrobial agents as *cyt b* mutation-based resistance is unlikely to develop without significant fitness cost to the target organism [48].

QoI/QiI binding may result in bathochromic shifts in the visible absorption spectra of reduced

cytochrome b_L/b_H [37], and this technique is convenient test for the predictions of *in silico* modelling approaches. The b_L -proximal inhibitors strobilurin and myxothiazol have been observed to induce 1–2 nm redshifts of haem b_L , shifting the absorption maximum of the α -band to 566 nm. A similar spectral shift is observed with the b_L -distal inhibitor stigmatellin (Fig. 1) [37]. At Q_i , the binding of the antagonist antimycin A induces a 2 nm bathochromic shift in the visible absorption spectrum of haem b_H , shifting the absorption maximum of the α -band to 564 nm. Relatedly, the phenomenon of ‘oxidant-induced reduction’ – the reduction in the low potential chain within *cyt bc*₁ in the presence of QiI and ascorbate or substrate quinol upon addition of an oxidant such as ferricyanide due to the bifurcated electron transfer chemistry at Q_o – can also provide clues as to the mode of action of *cyt bc*₁ Q -site inhibitors [11,49,50]. We recommend the use of these relatively simple spectrophotometric measurements, which can be performed with crude membrane preparations, as complementary to *in silico* binding predictions. Study of the enzymology of inhibition in Q_o/Q_i -site-directed mutants of yeast (and bacterial) *cyt bc*₁ can also provide useful information with regard to the mode of action of potential QoI/QiI [1,29,30,51].

Cytochrome *bc*₁ as a fungicide target in crop phytopathogens

Plant disease control is a major issue in agriculture. The fungicide market in Europe, for instance,

represents € 1.8 bn-year⁻¹; of this, € 1.3 bn-year⁻¹ is for wheat disease control [52]. A wide panel of fungicides is available with different modes of action and targets, such as the large group of fungal sterol biosynthesis inhibitors, the growing family of complex II inhibitors or the cyt *bc*₁ inhibitors.

The QoI fungicides and the rapid spread of target-site resistance

Cyt *bc*₁ is a successful target for agricultural fungicides, with most of the compounds in use being Q_o-site inhibitors (QoIs). They are (and have been) used to control a wide range of plant pathogen fungi belonging to the ascomycetes, basidiomycetes and oomycetes. Some of the pathogens are the causative agents of diseases of economically important crops, such as cereals and vines.

The QoIs available on the market include several synthetic strobilurins [53] such as azoxystrobin, dimoxystrobin, fluoxastrobin, kresoxim-methyl, picoxystrobin, pyraclostrobin, trifloxystrobin, but also the benzyl-carbamate pyribencarb, the oxazolidinone famoxadone and the imidazolone fenamidone. These compounds are likely to share the same binding mode as the resistance mutation G143A in the cyt *b* Q_o site confers cross-resistance to all of these inhibitors.

The first QoI marketed for agricultural use, azoxystrobin, was launched in 1996. Unfortunately, its efficiency was rapidly challenged within a few years, with resistant isolates reported in populations of pathogens, such as *Blumeria graminis* (wheat and barley powdery mildew) [54] and *Plasmopara viticola* (grape downy mildew) [55]. We will address additional fungal resistance mechanisms, namely the upregulation of alternative respiratory pathways and activity of membrane efflux pumps later in this review.

Fungal phytopathogen resistance to azoxystrobin was found to be caused by the cyt *b* mutation G143A in the Q_o site (Figs 1 and 3), and interestingly, A143 is found natively in cyt *b* of the strobilurin-producing basidiomycete *Mycena galopoda* [56]. The G143A substitution has been now found in isolates from over 25 species of phytopathogenic fungi [see Fungicide Resistance Action Committee (FRAC): www.frac.info]. As such, FRAC often recommend mixing two or more fungicides with differing modes of action to try and overcome this problem of resistance. The G143A mutation confers a high level of resistance that can result in a severe loss in disease control by QoIs. Inspection of the atomic structure of bovine cyt *bc*₁ with bound azoxystrobin indicates that the

replacement of G143 by alanine results in a destabilising steric clash with the MOA-bearing phenyl moiety of the fungicide. In yeast, G143A dramatically increases resistance to azoxystrobin (4000×) but has no effect on cyt *bc*₁ enzymatic activity [29], indicating that the binding of substrate ubiquinol is not hindered by the mutation. The high level of resistance without penalty on the enzyme activity – combined perhaps with a heavy use of QoIs in fields and thus a strong selection pressure – could explain the rapid emergence and spread of G143A in many fungi.

In some species, however, the exon–intron structure of the cyt *b* gene prevents the appearance of the G143A mutation [57]. In these species, an intron is located immediately after the codon for G143. When the G143-encoding codon GGT is located at the exon/intron boundary and is replaced by codon GCT (encoding alanine), the intron splicing is altered, resulting in a severe decrease in the amount of mature mRNA. Accordingly, the resulting loss of cyt *b* due to mutation at this mRNA splice junction has been demonstrated in the yeast model [58]. G143A would thus have a deleterious effect in the intron-containing species, affect the fitness of the resistant cells and be counter-selected in field.

Three other cyt *b* mutations have been reported in isolates resistant to QoIs – including ‘G143’ intron-containing species. F129L, found for instance in *Plasmopara viticola* and *Pyrenophora teres* (Barley net blotch) [59] [and G137R, such as is found for in isolates of *Pyrenophora. Tritici-repentis* (wheat tan spot) and G137S, found in *Venturia effusa* (pecan scab)] (Figs 1 and 3) [59,60].

G137 is at the N-terminal region of helix cd1 and is close to the interfacial region for ISP-ED binding. Substitution of glycine by arginine, a bulky and cationic residue, is expected to lead to local distortion of the loop connecting helices C and cd1. In yeast, the mutation causes in a significant defect in respiratory growth and cytochrome *bc*₁ activity [61]. In pathogenic fungi, the nonconservative substitution may be expected to result in a fitness penalty, which could explain the rare occurrence of G137R/S in the field [59,60].

The side chain of F129 is oriented towards the hydrophobic cavity that facilitates substrate access to Q_o. The residue is involved in binding the hydrophobic tail of stigmatellin and also presumably that of ubiquinol [62]. In yeast, the F129L mutation has little effect on the enzyme activity but decreases its sensitivity to QoIs azoxystrobin and stigmatellin [29]. F129 participates in side-chain van der Waals associations with these inhibitors in the respective cytochrome *bc*₁

structures, and the F129L substitution removes stabilising aromatic–aromatic interactions between the protein and bound azoxystrobin. F129L is not as widely spread in plant pathogen fungi as G143A and confers a moderate level of resistance.

G143A, now widespread in fungi in the field, is thus a major problem as the mutation confers high level of resistance and cross-resistance to the whole family of QoIs in use, compromising their efficiency in disease control. As such, new compounds are needed that either target the Q_i site or bind at Q_o but in a different mode, circumventing G143A-mediated resistance.

Metyltetraprole is a novel QoI that is not affected by the resistance mutation G143A [63]. It is active against ascomycetes, for instance *Zymoseptoria tritici* (wheat leaf blotch), a major threat for wheat production in western Europe. The compound has side chain similar to that of the synthetic strobilurin pyraclostrobin, but has a unique tetrazolinone moiety. It is suggested that this tetrazolinone moiety will not form the same highly specific interactions with the Q_o site as do other strobilurin-based QoIs and in consequence can accommodate changes in the target, such as G143A [64]. Thus, the steric clash due to G143A that can compromise the binding of QoIs would be limited by the unique size and shape of the tetrazolinone. The atomic structure of wt and G143A cytochrome *bc*₁ with bound metyltetraprole would be needed to confirm its distinct binding interactions with the target.

Oil fungicides in use and under development, and appearance of resistance mutations

To date, only three QiI fungicides have been commercialised and are oomycete-specific, namely the dimethylsulfonamide amisulbrom and cyazofamid, and the triazolopyrimidine ametoctradin.

In the FRAC repertory of fungicides (www.frac.info), ametoctradin was listed as QoSI, that is binding at the Q_o site in a manner similar to stigmatellin but distinct to QoIs [6]. Further spectroscopic studies indicated that the compound could target both Q_o and Q_i sites [31] and the fungicide was re-classified as QioI (<https://osf.io/qwg42/>). However, the appearance of the ametoctradin resistance mutation S34L in the Q_i site of *Plasmopara viticola*-resistant isolates showed that the fungicide preferentially targets the Q_i site, which was confirmed by the study of the mutation in the yeast model [51,65]. Analysis of *in silico* docking of ametoctradin into a homology model of the Q_i site of *Plasmopara viticola* suggests a binding mode similar to that of ubiquinol in yeast *cyt bc*₁. The aliphatic

octyl and ethyl substituents of ametoctradin are predicted to form stabilising hydrophobic interactions with the side chains of L17 (helix A), V194 and L198 (C-terminal region of helix D) (Fig. 3). The interactions between the amino-substituted triazolopyrimidinyl headgroup of the fungicide and the Q_i site appear more hydrophilic, with a putative H bond with the carboxylate side chain of residue D229. A weaker H-bonding interaction between this amino moiety of ametoctradin and the serinyl side chain of S34 is also predicted, which would be lost when serine is replaced by leucine. In addition, the bulky leucine is expected to sterically destabilise the fungicide binding, which would explain the resistance [31,51].

A cyazofamid resistance mutation was reported in field isolates of *Plasmopara viticola*. Interestingly, the mutation is short sequence duplication of six nucleotides resulting in the insertion of two residues E203-DE-V204 [66], located in the linker region between helices D and E, proximal to the benzoquinone headgroup of Q_i-site-bound quinone in yeast *cyt bc*₁. The insertion is likely to perturb the local fold around this region, and interfere with cyazofamid access or binding into the Q_i site.

Fenpicoxamid is a new QiI active against a broad range of ascomycetes, such as the wheat pathogen *Z. tritici*. It is still under commercial development. Fenpicoxamid is derived from UK-2A, a natural product of *Streptomyces* sp. that is structurally related to antimycin [67]. In *Z. tritici*, fenpicoxamid is readily converted into UK-2A by removing the isopropylcarboxymethylether group [68]. UK-2A is 100-fold more potent than fenpicoxamid in inhibiting *cyt bc*₁ [69]. Using the yeast model, three cytochrome *b* mutations causing fenpicoxamid resistance were identified, L198F and G37C and N31K [69]. Notably, mutations of these residues L198, N31 and G37 have been reported to cause resistance to different Q_i-site inhibitors in different organisms. Residue G37, in particular, seems a hot spot of resistance mutations [51].

The binding of UK-2A to yeast *cyt bc*₁ has been modelled *in silico* [69]. The results suggested a structural overlap between UK-2A and antimycin A in the Q_i binding pocket, with differences, in particular the pyridine head of UK-2A, is flipped by 180°. In this model, the N atom in the pyridine ring is protonated and can form a salt bridge with the carboxyl group of D229, a key residue in Q_i-site inhibitor binding. However, in contrast to UK-2A, other Q_i-site inhibitors form a hydrogen bond with an O atom of D229. It is likely that the local electrostatic environment around D229 is affected by the replacement of the nearby residue N31 by lysine (N31K), disrupting the salt bridge

(or bound water molecules, which may also interact with the Q_i antagonist [69]) and in consequence weakening the inhibitor binding. Destabilising steric hindrance is likely to explain the resistance caused by G37C and L198F, as the substitutions result in bulkier residues.

Alternative QoI/Qil resistance mechanisms in fungal phytopathogens

In addition to mutations in the inhibitor binding site within *cyt b* that account for most of the cases of reported resistance, other mechanisms may induce resistance towards QoIs and QiIs, such as increased efflux of fungicides and activation of a mitochondrial alternative oxidase (AOX) [70–72]. These mechanisms need to be taken into account in the strategies for monitoring and controlling the development of resistance.

Increased drug efflux is less prevalent and result in lower resistance level than *cyt b* mutations. It is caused by the upregulation of membrane transporters. As these efflux pumps can often transport different compounds, their increased activity leads to multiple drug resistance (MDR). In *Z. tritici*, for instance, MDR was reported and found to be caused by the overexpression of the transporter MFS1, induced by insertions in the promoter of its encoding gene [72,73].

AOX forms an alternative respiratory pathway within the mitochondria of the phytopathogen which may be upregulated when *cyt bc*₁ is inhibited. AOX is a single-subunit, (non-haem) di-iron containing monotopic membrane protein which functions as a nonprotonmotive quinol oxidase, which is widespread in plant, fungal and protist mitochondria (but notably absent from the *Plasmodium* sp.) [74–76]. The activity of AOX allows ubiquinol-linked respiration from NADH (via mitochondrial complex I) to oxygen, bypassing the inhibition of *cyt bc*₁, although at much reduced energetic efficiency. Four protons may be expected to be pumped into the IMS per 2 electrons transferred to oxygen in this alternative respiratory pathway due to the activity of protonmotive complex I alone (i.e. 4H⁺/2e⁻) [75]. This represents a significant decrease from the 10H⁺/2e⁻ yield expected for *cyt bc*₁/cytochrome *c* oxidase-linked respiration. Putatively AOX-linked fungicide resistance was first noted in laboratory-generated strains of *Z. tritici*, that were resistant to the (then) newly developed QoI azoxystrobin [77], and has subsequently been observed in QoI/Qil-treated field isolates of the phytopathogens *Plasmopara viticola*, *Z. tritici*, *Magnaporthe grisea* and *Mycosphaerella fijiensis* [65,78–80]. *In vitro* studies

suggest that, in the absence of selective pressure, AOX overexpression in *Plasmopara viticola* sporangia comes with an associated fitness penalty [6], presumably due to the decreased energetic efficiency of mitochondrial respiration as outlined above. Accordingly, the fungal preinfection stages of spore germination and host penetration may be unlikely to develop AOX-associated QoI/Qil resistance due to the ATP demand of these processes [53,70,77,80]. *In vitro*, AOX activity can be inhibited by the benzhydroxamic compound salicylhydroxamic acid (SHAM), a presumed competitive inhibitor for ubiquinol. SHAM is a relatively poor inhibitor of the AOX, with an inhibition constant (*K*_i) of 21 μM against the *Trypanosoma brucei* enzyme (as measured by glycerophosphate-linked oxygen uptake assay) [81]. As an antifungal agent, SHAM has also been observed to act synergistically with QoIs *in vitro*, but this response is species (and QoI)-dependent [65,70,82]. Unfortunately, SHAM is a relatively non-specific inhibitor and poorly taken up by plants, and so is unsuitable for use in the field [70]. It must also be noted that the native AOX activity is important for maintaining redox (and metabolite) homeostasis in plants, and inhibition of this enzyme may be deleterious under osmotic and light stress conditions [83,84]. Nevertheless, the intriguing possibility exists of the development of dual fungal *cyt bc*₁/AOX inhibitors, as compounds such as the quinoline aurachins, produced by the myxobacterium *Stigmatella aurantiaca*, are inhibitors of both enzymes [85–88]. We note also the potential for complex I/*cyt bc*₁ inhibition for fungicidal activity as Δ*p* would be expected to be severely diminished under such circumstances, regardless of the activity of AOX. Complex I-targeting fungicides are not in widespread use, although the methylpyrimidin-4-amine compound diflumetorim is approved for use in Japan for the treatment of rust and powdery mildew infection of ornamental crops [89].

The QoI alkoxy amino acetamide fungicide SSF-126 has been reported to increase superoxide production in *M. grisea* [90], and although the Q_o site of *cyt bc*₁ is the likely source of origin (presumably SSF-126 is acting as a b_L-proximal inhibitor in this instance, or there is cross reactivity of this compound with Q_i), this has not been tested directly. AOX expression was observed to be strongly induced on treatment with SSF-126, although interestingly the addition of exogenous hydrogen peroxide was also observed to increase AOX mRNA transcript levels [78]. Earlier studies reported a possible ROS-associated link with AOX expression in the yeast *Hansenula anomala* (syn. *Pichia anomala*) [91], and more recently, in tomato leaves [92]. The signalling pathway controlling this mechanism is

unknown, but it potentially may be exploited in the development of future *cyt bc*₁-targeted fungicides (by, for instance, stimulating mitochondrial catalase or peroxidase expression) to minimise AOX-associated resistance mechanisms.

The significance of phytopathogen AOX activity with regard to QoI/Qil fungicide resistance in the field has been debated [6,70]. Its increasing prevalence, however, warrants close attention, particularly as this respiratory shunt (and MDR activity, as described above) may facilitate the development of QoI/Qil-resistant *cyt b* mutants [65].

Finally, we note the potential of the new, bifunctional ‘hybrid’ antifungal agents under development that combine strobilurin and aromatic amide pharmacophores. Such compounds are thus capable of targeting both *cyt bc*₁ and succinate dehydrogenase (an additional antipathogenic target). These hybrid compounds have demonstrated notable efficacy against the plant pathogens *Pyricularia oryzae* and *Sclerotinia sclerotiorum* under laboratory conditions [93].

Cyt *bc*₁ as a chemotherapeutic target of the human malaria parasite

The cytochrome *bc*₁ complex of the human malaria parasite *Plasmodium falciparum* has a number of unique features that enable drug selectivity in humans. Notably, a four-residue deletion in the cd2 helix (Fig. 3) of plasmidial *cyt b*, a region of the protein in close proximity to key structural components of the Q_o site and the mobile domain of the ISP may modify the conformation of Q_o in the parasite, forming an exploitable element for antagonist selectivity. Sequence differences between Plasmodia and humans in the C-terminal region of the E-ef loop of *cyt b* (notably the loss of histidine and lysine residues in the parasite), a region of the protein adjacent to catalytically essential ef helix element of Q_o, may also help drive drug selectivity [2,94,95].

Besides contributing to the mitochondrial Δp [76,96–98], the quinol oxidase function of *cyt bc*₁ performs an important role within *Plasmodium* mitochondria, oxidising the electron acceptor pool for DHODH-mediated pyrimidine biosynthesis. As noted previously, the mitochondria of *Plasmodium* sp. lack the AOX found in plants, fungi and other protists, and so are reliant upon *cyt bc*₁ activity for this function [76]. During the intraerythrocytic stage of parasite development within the human host, provision of quinol oxidase function is believed to be essential for parasite survival. Consistent with this, inhibition of *cyt bc*₁ results in an increase in carbamoyl-aspartate and a reduction in

UTP, CTP and dTTP [3,99–101]. Further evidence of an essential link between mitochondrial function and pyrimidine biosynthesis is supported by the generation of an atovaquone-resistant phenotype in transgenic *Plasmodium falciparum* parasites expressing ubiquinone-independent yeast DHODH [102]. Inhibition of *Plasmodium cyt bc*₁ has been shown to affect the conversion of fumarate to aspartate, further linking mitochondrial function with pyrimidine biosynthesis and also possibly purine metabolism [103].

Inhibition of *Plasmodium falciparum cyt bc*₁ during the intraerythrocytic (blood) stages of parasite development result in a relatively slow death phenotype compared with other antimalarials such as artemisinin and semisynthetic derivatives thereof [3,104,105]. This feature appears to be consistent with other mitochondrially acting antimalarials and is possibly due to the drug acting only on late trophozoites and not on the earlier ‘ring’ stages [3,106,107]. Inhibition of *Plasmodium falciparum cyt bc*₁ has also been validated against liver stages of the malaria parasite, resulting in the utility of any developed inhibitors as prophylactic agents; however, inhibition of the parasite *cyt bc*₁ is not believed to be active against ‘dormant’ *P. vivax/ovale* hypnozoites and therefore not suitable for potential radical cure of relapse malaria [108,109].

Development and pharmacology of the *cyt bc*₁ Q_o inhibitor atovaquone

Atovaquone (Fig. 4) is the prototypal inhibitor of the *Plasmodium cyt bc*₁ that was successfully developed and registered for clinical use [110]. Currently, atovaquone is used as a fixed-dose combination with proguanil (Malarone) for the treatment of children and adults with uncomplicated malaria or as a chemoprophylactic agent for preventing malaria in travellers [108,111]. In recent years in the United States, Malarone has been estimated to account for *ca.* 70% of all antimalarial pretravel prescriptions [112]. Atovaquone is the product of over 50 years of research-intensive efforts to develop a safe and effective antimalarial [110]. Much of what is known of the essentiality of the parasite *cyt bc*₁ target and the physiological role of *cyt bc*₁ in mitochondrial function and linked biosynthetic pathways, for example the pyrimidine pathway, is through the use of atovaquone.

Atovaquone binding to *cyt b* was initially hypothesised based on studies performed on model organisms and molecular modelling. These studies, which include electron paramagnetic resonance spectroscopy of the Rieske [2Fe2S] cluster, site-directed mutagenesis of model organism *cyt b* and gene sequencing of

atovaquone-resistant *Plasmodium* species, demonstrate that atovaquone is most likely a competitive inhibitor of the parasite's cyt *b* Q_o site [1,2]. These studies were further supported by the demonstration using the X-ray structure of mitochondrial cyt *bc*₁ from *S. cerevisiae*, of atovaquone bound in the *b*_L-distal region of the Q_o site, at 3.0-Å resolution [4]. This study again confirmed the critical role of a polarised H bond to His181 of the extrinsic domain of the Rieske protein that interacts with the ionised hydroxyl group of the drug. The side chain of PEWY residue Y279 (Figs 1 and 3) provides an important (and orienting) aromatic–aromatic interaction with the hydroxynaphthoquinone moiety of the drug, stabilising its binding within Q_o. The carbonyl groups of the Q_o-bound atovaquone molecule do not appear to be directly involved in H-bonding associations with the polypeptide backbone of cyt *b*, with the glutamyl side chain of E272 pointing away from the drug, and towards haem *b*_L, in a manner reminiscent of that for the interaction with HDBT [36].

Malarone drug failure has been associated with atovaquone resistance, specifically with a missense point mutation at position 279 in cyt *b* (yeast notation, corresponding to 268 in the *Plasmodium falciparum* protein sequence), exchanging tyrosine for serine or cysteine (Y279S/C) or, less frequently, asparagine (Y279N) [1,113–117].

Position 279 in cyt *b* is highly conserved across all phyla and is located within the 'ef' helix component of the Q_o site (Figs 1 and 3), which is putatively involved in ubiquinol binding. The resultant atovaquone-resistant growth IC₅₀ (half-maximal inhibitory concentration) phenotype of these mutants is

some 1000-fold higher than susceptible strains; however, this is accompanied by a ~40% reduction in the *V*_{max} of cyt *bc*₁, suggestive of a significant fitness cost to the parasite [48]. We note that the Y279S mutation in *Plasmodium falciparum* confers weak cross-resistance to Q_o *b*_L-proximal inhibitor myxothiazol *in vitro* [48] (although this naturally occurring antibiotic is unsuitable for use as an antimalarial agent). Interestingly, the G143A resistance mutation, as discussed earlier, and so prevalent in fungal phytopathogens, has yet to be observed in laboratory or field isolates of this organism.

Atovaquone monotherapy gives rise to *de novo* resistance very rapidly [118,119]. The underlying reason for this phenomenon has not been determined but pharmacodynamic/pharmacokinetic considerations as well as the multiple copy number of mtDNA (*ca.* 30 in *Plasmodium falciparum* and up to 150 in *P. yoelli* [120]) and the effect of an increased mutation rate of mitochondrially encoded genes have been discussed as potential contributing factors [110]. Whilst *de novo* resistance to atovaquone can occur rapidly in the blood stages of *Plasmodium falciparum*, malaria transmission studies suggest that resistant parasites harbouring specific cyt *b* mutations are not able to complete their development in the mosquito and are therefore unlikely to spread in the field [121].

A further notable recent development has been in the formulation of atovaquone slow-release strategies for chemoprotection. In rodent malaria models, atovaquone solid drug nanoparticles have been demonstrated to confer long-lived prophylaxis against malaria [122]. Pharmacokinetic–pharmacodynamic analysis indicates that if translated to humans, this

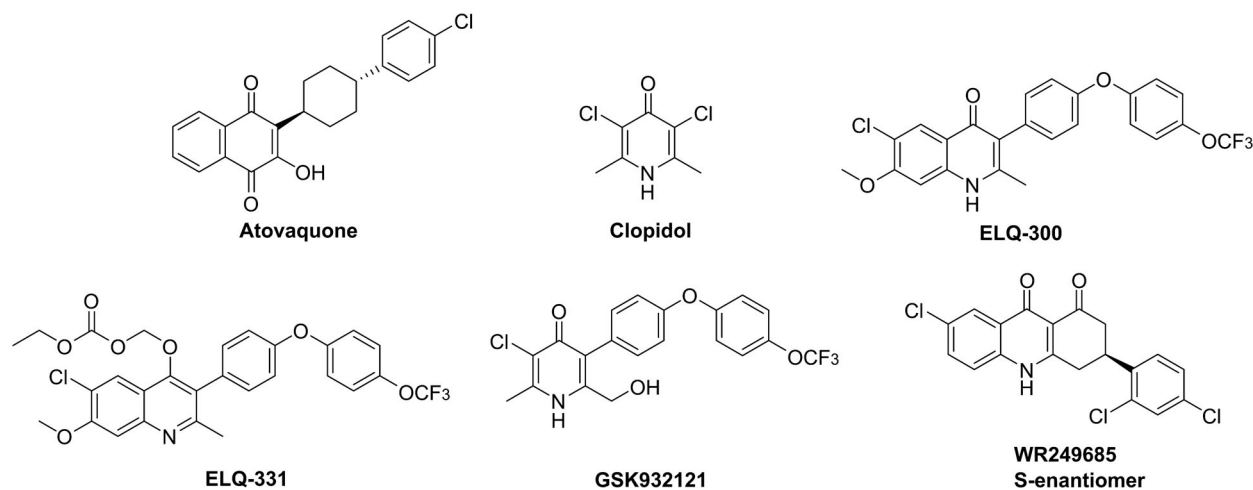


Fig. 4. Structures of selected antimalarial compounds in use and under development discussed in the text.

could potentially result in protection for at least one month after a single administration.

Development of second-generation *Plasmodium falciparum* cyt *bc*₁ inhibitors: pyridones, acridinediones, acridones and quinolones

Whilst the development of atovaquone gave rise to a new antimalarial therapy, its suboptimal efficacy when used for malaria treatment (versus prophylaxis), the cost and complexity of synthesis resulting in a high cost of goods, as well as the observed rapid emergence of parasite resistance, limited its potential use for the treatment of patients in malaria-endemic settings [110]. However, the malaria parasite cyt *bc*₁ offered a rare opportunity to drug developers as one of the few known validated drug targets resulting in the development of several inhibitor chemotypes.

Pyridones have been shown to possess antimalarial activity since the 1960s with the demonstration that clopidol possessed antimalarial efficacy against chloroquine-resistant parasites [2]. In the early 2000s, GSK Pharmaceuticals reported the preclinical development of pyridones targeting cyt *bc*₁ of *Plasmodium falciparum* displaying activity against atovaquone-resistant parasites [123]. Development of pyridones by GSK containing an atovaquone-like bicyclic side chain gave rise to a clinical candidate GSK932121 (Fig. 4) which entered phase 1 clinical trials in 2008 [124]. However, the trial was suspended due to the concurrent discovery of cardiotoxicity in animal studies dosed with the phosphate ester of the drug [125]. The toxicity was attributed to higher systemic exposure of the parent drug which was later confirmed when similar toxicity was observed in rats dosed with the parent drug by the intraperitoneal route. A subsequent study in which bovine cyt *bc*₁ was cocrystallised with the GSK932121 and other 4(1H)-pyridone class of inhibitors demonstrated that these inhibitors do not bind at the Q_o site but bind at the Q_i site, thereby providing an explanation for the apparent activity against atovaquone-resistant parasites (harbouring Q_o-site mutations) [5]. However, the study also demonstrated a much lower therapeutic index of the pyridones against bovine cyt *bc*₁ compared with atovaquone, thereby also providing a molecular explanation for the cardiotoxicity and eventual failure of GSK932121 in the phase 1 clinical trial.

Nevertheless, the Q_i site of *Plasmodium falciparum* cyt *bc*₁ offers itself as a promising target for drug development, particularly in combination with a Q_o-directed antagonist, such as atovaquone. We present a

protein sequence alignment of the cyt *b* Q_i site from *Plasmodium falciparum* in comparison with yeast and selected vertebrates in Fig. 5. This figure also highlights residues in the atomic structure of bovine cyt *b* (PDB accessions 1NTK, 4D6T and 4D6U in hydrogen-bonding or hydrophobic (side chain/ligand) interaction with antimycin and the 4(1H)-pyridone-class inhibitors GW844520 and GSK932121 [5,35]. The N-terminal region of transmembrane helix E within *Plasmodium falciparum* cyt *b* Q_i appears to offer considerable sequence diversity compared with mammalian counterparts, which may form exploitable differences for drug development. In particular, we highlight the single residue deletion in the loop connecting helices D and E, and the presence of aliphatic for ionic side-chain substitutions at L206, L217 and K220 (*Plasmodium falciparum* notation) for aspartate, lysine and leucine, respectively, in the human sequence data at the N terminus of transmembrane helix E. Y211/N213 in the parasite sequence in the same region also appear to offer significant steric (and electrostatic) diversity from the His/Tyr dyad at the equivalent position in the human sequence. Given the considerable challenge of obtaining an atomic structure for *Plasmodium falciparum* cyt *bc*₁, it is likely that yeast cyt *b* site-directed mutants may provide suitable surrogates for the study of antagonist binding at Q_i.

Drugs based on the acridine chemotype have a long history in malaria chemotherapy, indeed mepacrine was the first synthetic antimalarial blood schizontocide used clinically [126] and the related drug pyronaridine is still in use today in the form of Pyramax (pyronaridine–artesunate combination). Additional acridine derivatives include the acridones and the dihydroacridinediones, all of which display potent antimalarial activity for example [127]. Whilst the antimalarial efficacy of acridine congeners has been shown to derive from their ability to bind haem and thereby interfere with the parasite process of haem crystallisation [94,128–132], some potent dihydroacridinediones and acridones have been demonstrated to inhibit malaria parasite O₂ consumption [133] and specifically inhibit parasite cyt *bc*₁ [94,127]. Experiments performed with yeast manifesting mutations in cyt *bc*₁ reveal that binding of dihydroacridinediones is directed to the quinol oxidation site (Q_o) of cyt *bc*₁ [94].

Quinolones have been studied extensively as *bc*₁-targeting antimalarials [2,95,134]. A comprehensive set of alkyl and alkoxy 4(1H)quinolones have been synthesised in an effort to determine the structure–activity relationship (SAR) for antimalarial efficacy against asexual *Plasmodium falciparum* parasites, including

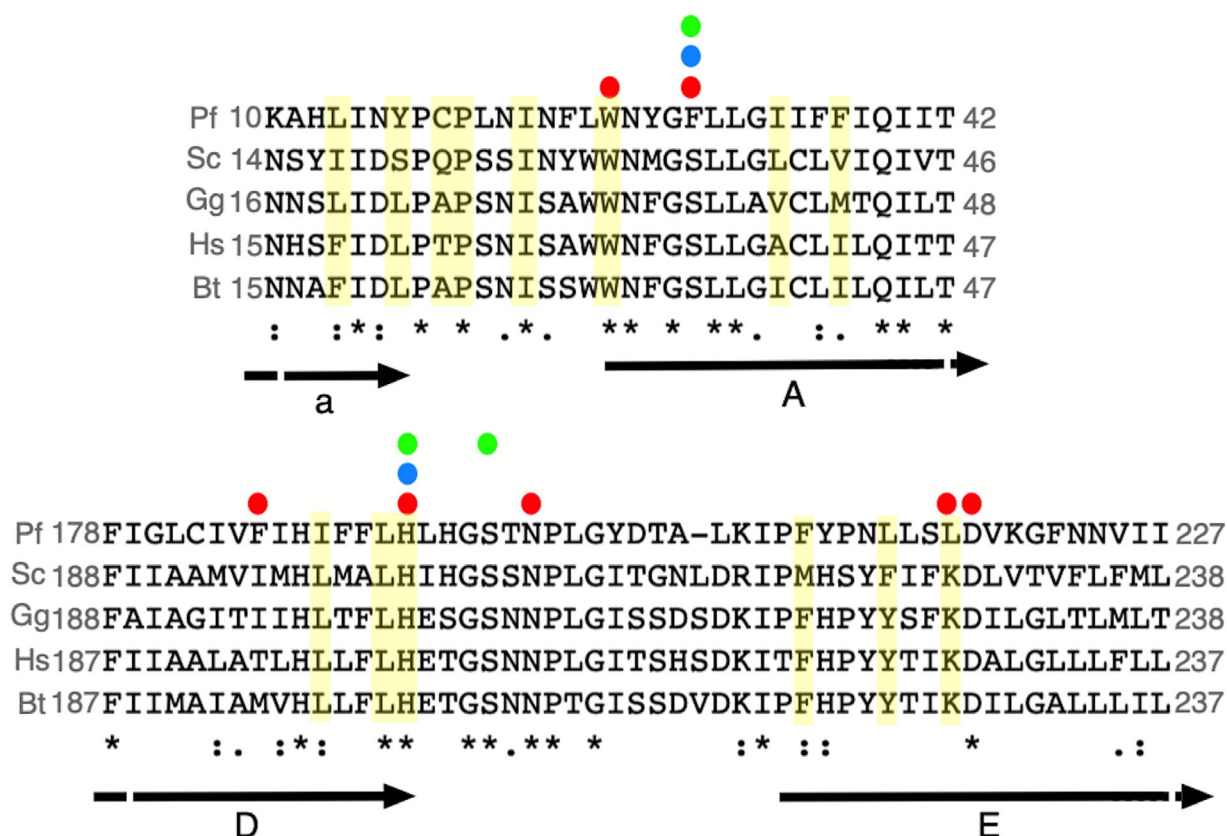


Fig. 5. Protein sequence alignment of the Q_i -site region of cytochrome *b* from *Plasmodium falciparum* (Pf, UniProtKB accession: Q02768), *Saccharomyces cerevisiae* (Sc, P00163), *Gallus gallus* (chicken, Gg, P18946), *Homo sapiens* (Hs, P00156) and *Bos taurus* (cow, Bt, PB00157). Sequence conservation in the aligned sequences is indicated using the CLUSTAL OMEGA convention [144], with asterisks, colons and periods indicating complete conservation, conservative substitutions and semi-conservative substitutions, respectively. Sequence data were obtained from the UniProt database. Arrows underneath the alignment indicate α -helices as identified in Fig. 3. Residues in the bovine cytochrome *b* atomic structures 1NTK [35], 4D6T [5] and 4D6U (*ibid*) in potential hydrogen-bonding association with bound antimycin, and the 4(1H)-pyridones GW844520 and GSK932121 are indicated by red, blue and green circles, respectively. Residues forming stabilising hydrophobic contacts with bound GW844520 in the bovine 4D6T structure are shaded in yellow. (GSK932121 displays similar binding interactions.) Protein–ligand associations indicated in this figure were predicted and analysed using the LPC software package [145].

atovaquone-resistant lines [135]. This work led to the discovery of ELQ-300 and P4Q-391 which were eventually selected as the preclinical candidate and backup molecule, respectively, by the Medicines for Malaria Venture (MMV) [136]. ELQ300 and P4Q-391 (Fig. 4) were developed based on endochin, which was discovered over 70 years ago [137]. However, whilst endochin and related alkyl-4(1H)quinolones possess antimalarial efficacy, the barrier to development was the metabolic instability due to the long alkyl chain. Replacement of the alkyl chain by the side chain from the previously described GSK pyridone series [124] proved to be a breakthrough for this chemotype as the molecules demonstrated improved metabolic stability whilst retaining antimalarial efficacy. Crucially, ELQ300 displays improved selectivity over human cyt

*bc*₁ relative to the withdrawn pyridone series [136]. Inhibition of the parasite cyt *bc*₁ is believed to be at the Q_i site (Fig. 5) [5], probably as a consequence of the QSAR being directed against atovaquone-resistant parasites harbouring Q_o -site mutations. Interestingly, *in vitro* isobole analysis of atovaquone and ELQ300 combination efficacy displays a synergistic interaction, suggesting that Q_o and Q_i inhibitors are a favourable combination strategy.

As outlined earlier, dual-site resistance may be expected to be unlikely to develop due to the increased probability of an associated organismal fitness penalty [48]. Additionally, inhibition at Q_i is likely to lead to increased (superoxide-linked) oxidative stress within the pathogenic organism [46]. (We also note that putative dual *Plasmodium falciparum* cyt *bc*₁/NADH

dehydrogenase inhibitors, such as the quinolone lead compound SL-2-25 [3] may also increase oxidative stress within the pathogen due to dehydrogenase-associated flavosemiquinone formation [138]. In addition, the Q_o-Y279S mutation does not confer appreciable resistance to this compound, nor to the related compound CK-2-68 [3,139].)

The ELQ class, however, does have some limitations, most notably the class suffers from poor aqueous solubility which *in vivo* is reflected in limited absorption and bioavailability – whilst this is not an issue in terms of reaching adequate *in vivo* exposures for efficacy, it is not possible to establish maximum tolerated doses to establish a therapeutic index. This issue stalled ELQ-300 development. However, more recently prodrug approaches are attempting to overcome this issue. ELQ-331, an alkoxy carbonate ester of ELQ-300, is currently the lead prodrug that is reported to have significantly increased ELQ-300 exposure after oral dosing [140]. ELQ-331 is currently advancing pre-clinical development as an oral formulation and is also being progressed as a long-acting injectable chemoprotection agent [141].

Other notable quinolone-based *cyt bc*₁ inhibitor developments include the identification of decoquinone [142] and quinolone esters [143], and these projects have thus far not developed further as they have not demonstrated superiority over existing molecules under development described above.

Cyt *bc*₁ as an antipathogenic target – general outlook and future prospects

The above discussion about atovaquone makes it clear that the development of parasite resistance is a concern for the continuing efficacy of *cyt bc*₁ Q_o-targeted antimalarial agents. However, this problem does not seem insurmountable, given the potential for the dual-site (Q_o/Q_i) combination therapy, or indeed, the development of true dual-site inhibitors, as proposed for the mode of action of the endochin compound ELQ-400 in yeast models of *Plasmodium falciparum cyt bc*₁ [32]. Furthermore, the lack of cross-resistance to lead compounds such as quinolone Q_o-antagonist CK-2-68 in the atovaquone-resistant *Plasmodium falciparum* strain TM90C2B [3] offers hope for the future exploitation of the Q_o site as an antimalarial chemotherapeutic target.

With regard to fungal phytopathogens, the question of QoI/QiI efficacy is even more pressing, in the light of the additional issues of the inducible AOX-mediated respiratory bypass and MDR efflux pumps. Nevertheless, and as with human malaria parasite, the

development of novel fungicidal QoI/QiI continues, particularly with the example set by the new QiI for the treatment of Ascomycete plant pathogens, fenpicoxamid [69], insensitive to G143A-mediated resistance. As above, dual-site (Q_o/Q_i) fungicidal activity would seem to offer the best chances against developing viable resistant strains, particularly if the issue of G143A resistance can be bypassed by the development of non-strobilurin-based inhibitors, occupying the *b*_L-distal region of Q_o. The recent exciting development of ‘hybrid’ bifunctional fungicides also deserves close observation [93]. Finally, we note that in the absence of suitable AOX inhibitors, the spread of respiratory bypass-based inhibition must be carefully monitored, and the necessity of the energetic role played by AOX during the various developmental stages of fungal growth.

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