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ACRIDINEDIONES: SELECTIVE AND POTENT INHIBITORS OF THE MALARIA PARASITE MITOCHONDRIAL bc_1 COMPLEX

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Running Title: Selective inhibition of malaria parasite bc_1 complex

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Non-standard abbreviations: TMRE; tetramethylrhodamine methyl ester; (Q_o) quinol oxidation site; (FCCP) carbonyl cyanide p-trifluoromethoxyphenyl hydrazone.

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Abstract

Clinical treatment-failures to affordable drugs have contributed to a global increase in the number of deaths arising from malaria infection. This unacceptable situation has stimulated research for new drugs active against multi-drug resistant *Plasmodium falciparum* parasites. In this regard, here we show that deshydroxy-1-imino derivatives of acridine (i.e. dihydroacridinediones) are selective antimalarials acting as potent (nM K_i) inhibitors of parasite mitochondrial bc_1 complex. Inhibition of the bc_1 complex lead to a collapse of the mitochondrion membrane potential ($\Delta\Psi_m$) resulting in cell death (IC_{50} ~15nM). The selectivity of one of the dihydroacridinediones against the parasite enzyme was some 5000 fold higher than for the human bc_1 complex, significantly higher (~200 fold) than that observed with atovaquone, a licensed bc_1-specific antimalarial drug. Experiments performed with yeast containing mutations in the bc_1 complex reveal that binding is directed to the quinol oxidation site (Q_o) of the bc_1 complex. This is supported by favourable binding energies for *in silico* docking of dihydroacridinediones to *P. falciparum* bc_1 Q_o. Dihydroacridinediones represent an entirely new class of bc_1 inhibitors and the potential of these compounds as novel antimalarials is discussed.

Introduction

Death and morbidity from malaria are on the increase largely as a result of parasite drug resistance(Snow et al., 2001). Consequently there are more people dying of malaria now than there were 20 years ago. Recognition of this problem by the international community and the engagement of the pharmaceutical industry and other key stakeholders, has catalysed the concerted search for new antimalarial drugs with novel targets (Biagini et al., 2005; Biagini et al., 2003; Edwards and Biagini, 2006).

Acridine-based drugs have a long history in malaria chemotherapy. Mepacrine was the first synthetic antimalarial blood schizontocide used clinically (Wernsdorfer and Payne, 1991) whilst the related drug pyronaridine, has been used for nearly 20 years as a monotherapy to treat malaria in China (Shao, 1990). More recently PYRAMAX®, a pyronaridine-artesunate combination treatment is currently undergoing phase III clinical trials (mmv.org). In addition, acridine congeners including the acridones (Basco et al., 1994; Winter et al., 2006) and dihydroacridinediones (Dorn et al., 2001; Durckheimer et al., 1980), have also demonstrated potent antimalarial activity, in some cases with good *in vitro* therapeutic indices (Winter et al., 2006).

Many acridine-based compounds can bind to heme (e.g. (Auparakkitanon et al., 2006; Auparakkitanon et al., 2003; Chou and Fitch, 1993; Dorn et al., 2001; Dorn et al., 1998)), the by-product of parasite haemoglobin digestion. Clinically relevant acridines such as quinacrine and pyronaridine are believed to confer almost all of their antimalarial activity through this interaction, by preventing the crystallization of heme (Auparakkitanon et al., 2006; Dorn et al., 1998). However not all acridine-based inhibitors kill the parasite via this route. Some 9-aniloacridines for example have been shown to exert their antimalarial activity through the inhibition of DNA topoisomerase II (Auparakkitanon and Wilairat, 2000; Gamage et al., 1994).

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Whilst some dihydroacridinediones have been reported to inhibit the malaria parasite respiratory pathway, causing a reduction in whole cell O_2 consumption (Suswam et al., 2001).

A role for parasite mitochondria is implicated by a degree of cross resistance between some dihydroacridinediones and atovaquone (Suswam et al., 2001), although this relationship is not conclusive and has not been demonstrated with allelically exchanged parasite lines. Furthermore, studies performed on beef-heart mitochondria revealed that alkyl-acridones inhibit a number of mitochondrial functions including Complex I (Oettmeier et al., 1992), Complex III (Oettmeier et al., 1994) (bc_1 complex, the membrane-bound complex containing two distinct quinone binding sites, Q_o (quinol oxidation) and Q_i (quinone reduction)) and the ATP/ADP translocase (Oettmeier et al., 1995). By virtue of their structural similarity to alkyl-acridones, the potent (pM) antimalarial activity of newly synthesised haloalkoxyacridones (Winter et al., 2006) has been attributed to inhibition of mitochondrial bc_1 complex, although it is possible that for some of these compounds antimalarial activity is a consequence of heme-binding.

In this study, we have investigated the antimalarial mode of action of two dihydroacridinediones, floxacrine and WR249685 (the S enantiomer of WR243246), developed by the Walter Reed Army Institute of Research (Fig. 1 (Dorn et al., 2001; Kesten et al., 1992; Raether and Fink, 1979; Raether and Fink, 1982; Schmidt, 1979)). Both of these compounds show heme binding and bc_1 inhibitory properties however, whilst floxacrine kills parasites via a heme-mediated process, WR249685 is shown here to be a highly selective inhibitor of the Q_o of the *P. falciparum* bc_1 complex. The molecular nature of the selectivity of these drugs and their potential as novel antimalarial drugs is discussed.

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Materials and Methods

Parasite, culture, and drug sensitivity assays. *P. falciparum* (3D7 strain) cultures consisted of a 2 % suspension of O+ erythrocytes in RPMI 1640 medium (R8758, glutamine, and $NaHCO_3$) supplemented with 10 % pooled human AB+ serum, 25 mM HEPES (pH 7.4), and 20 µM gentamicin sulfate (Trager and Jensen, 1976). Cultures were grown under a gaseous headspace of 4% O_2, 3% CO_2 in N_2 at 37°C. Parasite growth was synchronized by treatment with sorbitol (Lambros and Vanderberg, 1979). The sensitivity of *P. falciparum*-infected erythrocytes to various drugs was determined using the [^{3}H]-hypoxanthine incorporation method (Desjardins et al., 1979) with an inoculum size of 0.5 % parasitemia (ring stage) and 1% hematocrit. IC_{50}s were calculated by using the four-parameter logistic method (Grafit program; Erithacus Software, U.K.). To determine whether the antimalarial activity of two drugs is additive, antagonistic or synergistic, parasite growth was tested by titration of the two drugs at fixed ratios proportional to their IC_{50}s. The fractional inhibitory concentrations of the resulting IC_{50}s were plotted as isobolograms (Berenbaum, 1978).

Inhibition of in vitro hemozoin formation. Assays were performed based on the methods by Bray et al. (Bray et al., 1999) and Stead et al., (Stead et al., 2001). Briefly, an aliquot of ghost erythrocyte membrane (100 µl) and FPIX (100 µl of 3 mM in 0.1 M NaOH) were mixed with an aliquot of 1 M HCl (10 µl) and sodium acetate (500 mM, pH 5.2) was added to give a volume of 900 µl in each tube. A series of drug concentrations were prepared in water and 100 µl of each was added to the appropriate samples. Samples were mixed and incubated for 48 h at 37°C, with occasional mixing. After incubation, samples were centrifuged (15,000 g, 15 min, 21°C) and the hemozoin pellet repeatedly washed with 2% w/v SDS in 0.1 M sodium bicarbonate, pH 9.0, until the supernatant was clear (usually 3-4 times). After the final wash, the supernatant was removed and the pellet was resuspended in 1 ml of 0.1 M NaOH and incubated for a further 1 h at room temperature. The hemozoin content was determined by measuring the absorbance at 400. The

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concentration of drug required to produce 50% inhibition of hemozoin production (IC_{50}) was determined graphically as described for the drug sensitivity assays.

Determination of heme-drug dissociation constants. Heme-drug equilibrium constants were determined based on a UV-visible spectroscopic method (Egan et al., 1997). To provide a strictly monomeric heme (ferriprotoporphyrin IX) species in solution, heme (6 μM) was prepared in a HEPES (20 mM, pH 7.2) buffered solution of 40 % (v/v) DMSO (Egan et al., 1997). UV-visible titrations of antimalarial drugs chloroquine, amodiaquine, floxacrine and WR249685 were performed monitoring the Soret band of the porphyrin (390-460 nm). The resulting titration curves were analysed using a non-linear curve fitting programme (Pro-Fit) and thermodynamic parameters were derived from modelling, assuming a 1:1 complexation of drug and heme (Egan et al., 1997; Marques et al., 1996).

Preparation of P. falciparum cell-free extracts. Free parasites were prepared from aliquots of infected erythrocytes (approximately 8×10^9 cells ml^{-1}) by adding 5 volumes of 0.15% (wt/vol) saponin in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 1.76 mM K_2HPO_4, 8.0 mM Na_2HPO_4, 5.5 mM D-glucose, pH 7.4) for 5 min, followed by three washes in HEPES (25 mM)-buffered RPMI containing a protease inhibitor cocktail (Complete Mini; Roche). A cell extract was prepared by repeated freeze-thawing in liquid N_2, followed by disruption with a sonicating probe.

Human liver microsome preparation. Histological normal liver was obtained from Caucasian transplant donors. The certified cause of death was traumatic injury due to a road traffic accident. The liver samples were transferred to the laboratory within 30 min of death. They were portioned, frozen in liquid nitrogen, and stored at −80 °C. Approval was granted by the Liverpool local research Ethics Committee and prior consent was obtained from the donors' relatives.

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Liver tissue was washed briefly in ice-cold isolation buffer (0.154 M KCl, 50 mM Tris-HCl, pH 7.4). The tissues were homogenized in 4× volume of isolation buffer, and then centrifuged (10,000g, 20 min, 4°C). The pellet was discarded and the supernatant was then centrifuged (105,000g, 60 min, 4°C). The microsomal pellet was washed by resuspension in fresh buffer and centrifuged again (105,000g, 60 min, 4°C). Microsomes were resuspended in 2× volume of 0.12 M Tris, pH 7.4, and stored frozen (−80 °C) in 1 ml aliquots at 80°C.

Preparation of yeast cytochrome b mutants. Generation of mutant strains and preparation of crude mitochondrial membranes was performed as described previously (Fisher et al., 2004b).

Bovine mitochondrial membrane preparation. Bovine mitochondrial membranes (Keilin-Hartree particles) were prepared as described by Kuboyama et al (Kuboyama et al., 1972)

Rat liver microsome preparation. Adult male Wistar rats were obtained from Charles River Laboratories (Margate, Kent, UK). Wistar rat (RLM) liver microsomes were prepared from male rats (125-170g) as described by Gill et al., (Gill et al., 1995).

Preparation of decylubiquinol. The artificial quinol electron donor was prepared based on the method of Fisher et al., (Fisher et al., 2004b). Briefly, 2,3-dimethoxy-5-methyl-n-decyl-1,4-benzoquinone (decylubiquinone), an analogue of ubiquinone, was dissolved (10 mg) in 400 µl of nitrogen-saturated hexane. An equal volume of aqueous 1.15 M sodium dithionite was added, and the mixture shaken vigorously until colourless. The upper, organic phase was collected, and the decylubiquinol recovered by evaporating the

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hexane under N_2. The decylubiquinol was dissolved in 100 µl of 96 % ethanol (acidified with 10 mM HCl) and stored in aliquots at -80 °C. Decylubiquinol concentration was determined spectrophotometrically from absolute spectra, using $\varepsilon_{288\text{-}320} = 4.14\ mM^{-1}\ cm^{-1}$.

Measurement of bc_1 activity. Cytochrome *c* reductase activity measurements were assayed in 50 mM potassium phosphate (pH 7.5), 2 mM EDTA, 10 mM KCN, and 30 µM equine cytochrome *c* (Sigma) at room temperature (Fisher et al., 2004b). Cytochrome *c* reductase activity was initiated by the addition of decylubiquinol (50 µM). Reduction of cytochrome *c* was monitored in a Cary 4000 spectrophotometer at 550 versus 542 nm. Initial rates (computer-fitted as zero-order kinetics) were measured as a function of decylubiquinol concentration. The cytochrome *b* content of membranes was determined from the dithionite-reduced minus ferricyanide-oxidized difference spectra, using $\varepsilon_{562\text{-}575} = 28.5\ mM^{-1}\ cm^{-1}$ (Vanneste, 1966). Turnover rates of cytochrome *c* reduction were determined using $\varepsilon_{550\text{-}542}=18.1\ mM^{-1}cm^{-1}$ (Margoliash and Walasek, 1967).

Inhibitors of bc_1 activity were added without prior incubation. DMSO in the assays did not exceed 0.3 % (v/v). IC_{50}s were calculated using the four-parameter logistic method (Grafit program; Erithacus Software, U.K.). The equilibrium dissociation constant (K_i) of inhibitor binding to bc_1 was determined using the Cheng-Prusoff equation (Cheng and Prusoff, 1973).

Real-time single-cell monitoring of membrane potential ($\Delta\Psi_m$). The rhodamine derivative, TMRE (tetramethylrhodamine ethyl ester), was used to monitor the membrane potential ($\mathbf{\Delta}\Psi_m$) of the plasma-membrane and mitochondria of malaria infected red blood cells(Biagini et al., 2006). TMRE is cationic and reversibly accumulates inside energised membranes according to the Nernst equation. For experimentation, suspensions (1%) of infected erythrocytes in HEPES-buffered RPMI medium (no serum) were loaded with

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TMRE (250 nM, Molecular Probes) for 10 min at 37 °C. For imaging, malaria parasite-infected erythrocytes were immobilized using polylysine-coated coverslips in a Bioptechs FCS2 perfusion chamber and maintained at 37 °C in growth medium (no serum). Inhibitors were added to the perfusate, and the membrane potential-dependent fluorescence responses were monitored in real time. During all manipulations, the concentration of TMRE in the perfusate was kept at 250 nM. The fluorescence signals from malaria-infected erythrocytes were collected on a Zeiss Pascal confocal laser scanning microscope through a Plan-Apochromat 63x 1.2 N.A. water objective. Excitation of TMRE was performed using the HeNe laser line at 543 nm. Emitted light was collected through a 560-nm long pass filter from a 543-nm dichroic mirror. Photobleaching (the irreversible damage of TMRE producing a less fluorescent species) was assessed by continuous exposure (5 min) of loaded cells to laser illumination. For each experiment, the laser illumination and microscope settings (e.g. laser power both voltage settings and attenuation [%], scan speed, pinhole diameter, number of scan sweeps, and degree of magnification) that gave no reduction in signal were used. Data capture and extraction were carried out with Zeiss Pascal software and Photoshop.

Dihydroacridinedione docking into P. falciparum cytochrome b (Q_o). A predicted model of the *P. falciparum* cytochrome *b* of the bc_1 complex was constructed with SWISS-MODEL using bovine cytochrome *b* PDB coordinate sets 1ntmC, 1sqxC, 1l0nC, 1ntkC and 1be3c as the structural templates. *In silico* docking was performed using Autodock 3.05 (Morris et al., 1998) and associated suite of programs. Autodock uses an empirical scoring function to estimate the free energy of binding. This function contains five terms: A Lennard_Jones 12-6 dispersion/repulsion term; a directional 12-10 hydrogen bonding term; a screened Coulombic electrostatic potential; unfavourable entropy of binding due to restricted conformations and a desolvation energy term. For the calculations, the protein atoms were kept fixed with the inhibitors allowed full flexibility. A combination of a Lamarckian genetic algorithm and pseudo-Solis and Wets local search

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was used to generate docking poses for each molecule. The docking was performed using a grid much larger than either the Q_o or Q_i binding sites. The parameters used in this 'blind docking' procedure were those that have been shown to reproduce the binding mode of drugs within known structures of drug:crystal complexes with no prior knowledge of the binding site (Hetenyi and van der Spoel, 2002). The most favourable docking pose for each molecule was identified by the scoring function.

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Results

Dihydroacridinedione binding to heme. Assays were performed to determine the affinity of dihydroacridinediones for heme as well as to determine their ability to inhibit heme crystallization, relative to known heme-binding drugs. Floxacrine and chloroquine (CQ) were shown to inhibit heme crystallization with an IC_{50} of 63 and 56 μM, respectively. WR249685 inhibited hemozoin formation by 50 % at 130 μM, however complete inhibition was not measurable as this was the highest concentration achievable in the assay (values means of three independent determinations).

Heme-drug equilibrium constants were determined for CQ, amodiaquine (AQ), floxacrine and WR249685 by measuring the shift of the heme Soret band on titration of drugs. In buffered DMSO (40 % (v/v)) solutions, the heme-drug dissociation constants (K_i) were calculated to be; 1.38 μM for CQ, 1.55 μM for AQ, 1.87 μM for floxacrine and 31.74 μM for WR249685 (values means of two independent determinations).

The relative poor heme binding affinity of WR249685 was in contrast to its potent *in vitro* antimalarial activity (IC_{50} 15 nM) which was comparable to that for CQ (IC_{50} 7.4 nM), AQ (IC_{50} 4.5 nM) and significantly better than that for floxacrine (IC_{50} 140 nM, Table 1).

Dihydroacridinediones inhibit P. falciparum bc_1 activity. The ability of floxacrine and WR249685 to inhibit bc_1 complex activity was determined in a number of species and compared to that of well known bc_1 inhibitors (Fig. 1). For all species, bc_1 activity was determined by monitoring the reduction of cytochrome *c* with decylubiquinol (QH_2) as electron donor.

P. falciparum bc_1 activity exhibited Michaelis-Menten kinetics with an apparent concentration of substrate leading to half-maximal velocity (K_m) for QH_2 of 6.2 ± 2 μM reaching a maximum/limiting velocity (V_{max}) of

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576 ± 88 nmoles cytochrome *c* reduction s^{-1} mg^{-1} free parasite protein (Fig. 2a). Human bc_1 displayed similar saturation kinetics with a K_m for QH_2 of 7.8 ± 2 μM and a V_{max} turnover of 3 s^{-1} (Fig. 2b). Note that turnover (s^{-1}) for *P. falciparum* cell-free extracts could not be determined due to interference of free heme and hemozoin with cytochrome *b* determinations. Both *P. falciparum* and human liver bc_1 activities could be stimulated ≥10 fold by addition of surfactant (0.025% (w/v) dodecyl maltoside). However, in order to compare data with similar studies (Fry and Pudney, 1992), all inhibitory assays were performed in the absence of surfactant.

Inhibition profiles of bc_1 complexes from bovine heart, rat liver, human liver and *P. falciparum* are described in Table 1. The bc_1 complexes from all species were shown to be similarly sensitive to the well known bc_1 inhibitors stigmatellin, and myxothiazol. The antimalarial drug atovaquone displayed selective inhibition for *P. falciparum* bc_1 complex (IC_{50} 3 ± 2 nM, K_i= 0.3 nM) over mammalian bc_1 complexes. Interestingly however, human and bovine bc_1 activity was observed to be more sensitive to atovaquone (IC_{50} 72 and 83 nM, respectively) compared to rat liver bc_1 activity (IC_{50} 406 nM). Similarly, albeit more modestly, the pyridone GW844520, displayed selective toxicity for the parasite enzyme (IC_{50} 32 nM) over mammalian enzymes (IC_{50}'s ranging 51 – 353 nM). The dihydroacridinedione WR249685 was shown to be selective exclusively for *P. falciparum* bc_1 complex (IC_{50} 3 nM, K_i = 0.3 nM). Notably, the *in vitro* therapeutic index (TI) for this inhibitor against human bc_1 was >4600. This dramatic selectivity is significantly higher than that observed with atovaquone (TI = 24) or with the pyridone GW844520 (TI = 5). Floxacrine was shown to have moderate inhibitory activity against cross-species bc_1 activities (IC_{50}'s ranging 328-1458 nM), but without any selectivity over the *P. falciparum* enzyme.

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Inhibition of yeast bc_1 complex by dihydroacridinediones is specific to the quinol oxidation site (Q_o). The inhibitory profile of the dihydroacridinediones was determined against wild type and genetically engineered *Saccharomyces cerevisiae* harbouring cytochrome *b* mutations Y279S and G143A. The Y279S mutation corresponds to the Y268S mutation in *P. falciparum* cytochrome *b* Q_o exhibiting an atovaquone resistant phenotype (Fisher and Meunier, 2005; Korsinczky et al., 2000; Srivastava et al., 1999; Syafruddin et al., 1999). The G143A mutation confers dramatic resistance to heme proximal Q_o inhibitors such as myxothiazol (Fisher et al., 2004a; Fisher and Meunier, 2005). As expected, atovaquone was shown to have potent bc_1 inhibitory activity against wild type yeast (IC_{50} 3 nM) and G143A mutants (IC_{50} 27 nM), whilst the Y279S mutation conferred significant resistance (IC_{50} 2689 nM, Table 2). Interestingly, the Y279S mutation was also associated with a moderate increase in tolerance to the pyridone GW844520, floxacrine and WR249685 (Table 2). Taken together these data indicate that all of these inhibitors target the Q_o site of the bc_1 complex.

Dihydroacridinediones collapse mitochondrial membrane potential $\Delta\Psi_m$. The measurement of mitochondrial $\mathbf{\Delta}\Psi_m$ was based on the accumulation of the cationic fluorescence probe TMRE according to the Nernst equation. Addition of TMRE to *P. falciparum*-infected erythrocytes, resulted in a strong fluorescence signal originating from the parasite cytosol denoting the existence of a high $\mathbf{\Delta}\Psi_m$. This phenomenon has been observed previously(Biagini et al., 2006) and is the result of the high $\mathbf{\Delta}\Psi_m$ (−100 mV) of the plasma membrane (Allen and Kirk, 2004). Upon addition of the V-type H^+ ATPase inhibitors bafilomycin A_1 or concanomycin (200 nM), approximately 70 to 80% of the fluorescence signal was lost (not shown), leaving a small but strong signal originating from the parasite mitochondrion (Biagini et al., 2006).

Since both the plasma membrane and the mitochondrion $\mathbf{\Delta}\Psi_m$ contribute to the accumulation of TMRE, we could not accurately quantify the finite $\mathbf{\Delta}\Psi_m$ values. Therefore, for all experiments, the fluorescence dynamic range was set up so that untreated TMRE-loaded cells were regarded as having complete

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fluorescence (100%), while the baseline (0%) was set by addition of the protonophore FCCP (10 μM). For mitochondrial-dependent fluorescence, bafilomycin A_1-treated cells were normalized to 100% and, again, the baseline (0%) was set by FCCP (10 μM).

Addition of the dihydroacridinediones, WR249685 (Fig. 3a) and floxacrine (not shown), was observed to partially reduce (~20%) the total cellular $\mathbf{\Delta}\Psi_m$ –dependent TMRE fluorescence, possibly indicating an effect on the mitochondrial contribution. This view was confirmed by the reduction of mitochondrial $\mathbf{\Delta}\Psi_m$ –dependent fluorescence from bafilomycin A_1-treated parasites with WR249685 (Fig. 3b) and floxacrine (not shown).

*Drug combination analysis of atovaquone with WR249685***.** As the data indicate that both atovaquone and WR249685 target the bc_1 complex of *P. falciparum* mitochondrial ETC, it was of interest to determine whether the two inhibitors would compete for ligand binding resulting in antagonistic inhibition. To do this, we performed isobole analysis of growth inhibition by titration of the drugs at fixed ratios proportional to their IC_{50}'s. The resultant isobologram of atovaquone and WR249685 is suggestive of a possible antagonistic interaction (Fig. 4).

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Discussion

In this study, the mode of action of two potent antimalarial dihydroacridinediones has been investigated. Historically, acridine derivatives have been considered to owe their antimalarial activity to their ability to bind heme. Therefore we first investigated the relative heme binding properties of floxacrine and WR249685. Thermodynamic analysis of heme-binding revealed that both inhibitors could bind to heme with a rank order relative to other well known heme binding drugs of; CQ > AQ> floxacrine >> WR249685. The heme-drug equilibrium constants for CQ and AQ closely match those reported previously by other workers (Egan et al., 1997). CQ, floxacrine and to a lesser extent WR249685 were shown to be able to inhibit *in vitro* hemozoin formation. This data is consistent with that of Dorn et al., (Dorn et al., 2001), which showed a comparable rank order of inhibition of *in vitro* hemozoin formation, i.e. CQ > floxacrine >> WR243246 (the R enantiomer of WR249685). The relative poor heme binding affinity of floxacrine compared to CQ and AQ is reflected by the lower *in vitro* antimalarial activity, i.e. floxacrine IC_{50} 140 nM, compared to CQ IC_{50} 7.4 nM and AQ IC_{50} 4.5 nM. However, WR249685 demonstrated potent *in vitro* antimalarial activity (IC_{50} 15 nM) whilst displaying a >20 fold poorer affinity for heme compared to the other antimalarial drugs.

As concluded by Dorn et al., (Dorn et al., 2001), it appears that heme does not play a major role in the mode of action of all dihydroacridinediones. Given that related dihydroacridinediones have been shown to affect respiration of *P. falciparum* (Suswam et al., 2001), we next investigated whether the mode of action of WR249685 was related to the inhibition of respiratory components.

Mild cross resistance (4-9 fold) of a dihydroacridinedione (WR243251) has been described in *P. falciparum* strains displaying 8,700 – 23,000 increase fold resistance in atovaquone (Suswam et al., 2001). Although these parasite lines also displayed an increase in resistance to other antimalarials such as CQ, as the site of

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action of atovaquone is the bc_1 complex (Fry and Pudney, 1992), this respiratory component was chosen for investigation.

The bc_1 complex is a membrane bound enzyme catalysing the transfer of electrons from ubiquinol to cytochrome *c* coupled with the concomitant vectorial translocation of protons across the inner mitochondrial membrane (Crofts, 2004). The catalytic core of the enzyme is made up of cytochrome *b*, cytochrome c_1 and the Rieske iron-sulphur protein (ISP). The catalytic mechanism, known as the Q-cycle (Crofts, 2004; Mitchell, 1975) involves two distinct quinone bindinding sites within cytochrome *b*, the quinol oxidation site Q_o and the quinone reduction site Q_i (Crofts, 2004). These two sites are situated on opposite sides of the membrane linked by a transmembrane electron pathway via hemes b_l and b_h (Crofts, 2004). A number of inhibitors selective for bc_1 Q_o and Q_i sites have been developed over recent years, most notably to control crop and human pathogens (Crofts et al., 1999; Esser et al., 2004; Fisher et al., 2004a).

In our study, stigmatellin, which binds in the b_l distal domain of Q_o (close to the docking site of ISP)(Crofts et al., 1999) and myxothiazol, which binds in the b_l-proximal position (Crofts et al., 1999), were both shown to be potent broad spectrum bc_1 inhibitors (Table 1). Interestingly however, inhibition of bc_1 activity by the dihydroacridinediones, pyridone and naphthoquinone was highly species selective (Table 1).

Species selectivity was most notably demonstrated by WR249685, which displayed a K_i for *P. falciparum* of 0.3 nM and an *in vitro* therapeutic index (TI) against human bc_1 of >4600 (Table 1). Yeast carrying the Y279S mutation in cytochrome *b* (corresponding to the Y268S mutation in *P. falciparum* conferring atovaquone resistance (Fisher and Meunier, 2005; Korsinczky et al., 2000; Srivastava et al., 1999; Syafruddin et al., 1999)) were observed to be less sensitive to WR249685 suggesting that Q_o is the binding site for this

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inhibitor (Table 2). So what is it about the *P. falciparum* bc_1 Q_o site that lends it self to inhibition by WR249685?

X-ray crystallography has shown that the overall fold of the α-carbon backbone of cytochrome *b* is highly conserved in prokaryotic and eukaryotic organisms (Fig. 5a). However, despite the high degree of sequence and structural conservation, there are notable differences in key regions of the malaria parasite Q_o site. Significantly, a homology model of the *P. falciparum* cytochrome *b* (constructed with SWISS-MODEL using bovine cytochrome *b* atomic coordinates as the structural template) suggests that the four residue deletion in the cd2 helix results in a 13Å displacement of this structural element when compared to the mammalian enzyme (Fig. 5b). Similarly, the α-carbon atom of the N-terminal proline of the ef helix (containing the catalytically essential 'PEWY' motif) is predicted to be displaced by 2Å in comparison to the mammlian enzyme. Other important differences include the replacement of lysine(269) by valine and alanine(277) by phenylalanine in the *P.falciparum* ef helix, and the exchange of phenylalanine(140) for tyrosine in the cd1 helix. Docking of WR249685 (and floxacrine, not shown) to the *P. falciparum* bc_1 Q_o model was energetically favourable (binding energy -8.1 kcal/mol, Fig. 6a), and additionally the model demonstrated selectivity in the docking of classical Q_o and Q_i inhibitors (e.g. famoxadone and antimycin, data not shown).

Figure 6b shows Q_o site residues predicted to be within 4 Å of the bound WR249685 (most energetically favourable conformation). The interactions are predominantly hydrophobic, although a backbone hydrogen bond from Ser-241 to the aromatic secondary amine of WR249685 is likely to be important for the positioning of the compound at Q_o. The glutamyl sidechain of Glu-261 shows considerable mobility and may also be involved in weak dipolar interactions with the chlorine atoms of WR249685. The most striking

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feature of the model for WR249685 binding to *Plasmodium* cytochrome *b* is the putative association between the inhibitor and the E-ef linker region (residues 236-241) of the cytochrome (Fig. 5a, 6b), a region of low sequence identity between *Plasmodium* and mammalian cytochrome *b*. The E-ef linker has not previously been recognised as a component of the Q_o site in the elucidated bc_1 crystal structures, and thus may explain the very high degree of selectivity of WR249685 for the *Plasmodium* enzyme.

It is necessary however to be circumspect in the interpretation of the modelling data. It should be noted that 'structural' water molecules at Q_o were not included in the modelling process, and these may influence the binding energy and positioning of WR249685. Additionally, the Rieske ISP headgroup was omitted during the modelling process, which has two important consequences. Firstly, the loss of a potential hydrogen-bond donor to the Q_o site via [2Fe-2S] cluster ligand His-161 (Esser et al., 2004) and, secondly, the steric volume occupied by the ISP is absent, which may allow for non-physiological but otherwise energetically favourable *in silico* docking of bulky inhibitors at Q_o.

The 5.6-fold increase in IC_{50} for atovaquone in rat liver microsome preparations compared to the human equivalent (Table 1) is, at first sight, surprising given the sequence homology between these species in the cd1 and ef regions of cytochrome *b* (Fig. 5a). It is possible that this difference is due to slight variation in the local fold and protein environment around Q_o, but a minor change in hydrogen-bonding capacity in the C-terminal region of transmembrane helix C may also weaken the interaction with atovaquone, raising the binding energy required for a stable association. Notably, bovine and human cytochrome *b* possess a potenial Q_o-site hydrogen bond donor in the forms of Thr-121 and Thr-122 respectively, residues which are absent in rat. In addition, there is conservative variation in the aliphatic composition of the F1 helix between

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these three species, which may result in an unfavourable steric environment for atovaquone binding in rat cytochrome *b*.

In a similar fashion the pyridone GW844520, shown to be specific for the bc_1 Q_o site (Table 2), also displayed a 2 fold selectivity for human bc_1 over the rat enzyme (Table 1), with an *in vitro* therapeutic index (TI) against human bc_1 of only 5 (Table 1). The drug development of this particular pyridone was terminated in late 2005 by the Medicines for Malaria Venture (MMV) due to toxicity issues (www.mmv.org). Currently a new pyridone (GSK932121A) is being developed with reduced toxicity. It will be interesting to establish whether this compound has an improved TI against human bc_1.

To our knowledge this study is the first to report human liver bc_1 activity. At this stage we have no idea of inter-patient variation of bc_1 activities, nonetheless our data indicate that rat liver enzyme is a poor model for human bc_1 and therapeutic indices generated from rat liver data should be treated with a degree of caution.

Addition of dihydroacridinediones to malaria infected erythrocytes was shown to cause the depolarisation of mitochondrial $\Delta\Psi_m$ (Fig. 3). We hypothesise that the depolarisation of mitochondrial $\Delta\Psi_m$ leads to a loss mitochondrial function and parasite death. Given that during the intraerythrocytic stage of the malaria life cycle, the parasite relies mainly on fermentation of glucose, the essential role(s) of the mitochondrion is not known, but it probably includes orotate production for pyrimidine biosynthesis(Gutteridge et al., 1979; Hammond et al., 1985) and Ca^{2+} homeostasis (Gazarini and Garcia, 2004; Uyemura et al., 2000). Furthermore the close juxtaposition of the mitochondrion with the plastid suggests an interdependence for essential metabolism(Goodman et al., 2007; Kobayashi et al., 2007).

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Recently it has been reported that addition of atovaquone does not cause a depolarisation of parasite mitochondrial $\Delta\Psi_m$, as $\Delta\Psi_m$ is generated by the ATP synthase and adenine nucleotide translocator (ANT) operating in reverse (Painter et al., 2007). We however have questioned these conclusions (Fisher et al., 2008), and maintain that targeting the proton pumping bc_1 complex leads to a depolarisation of $\Delta\Psi_m$ resulting in a loss of mitochondrial function and parasite death. Thus in our opinion targeting the mitochondrial ETC leading to a depolarisation of mitochondrial $\Delta\Psi_m$ remains a viable chemotherapeutic strategy. The merit of this strategy is supported by recent evidence showing an up-regulation of parasite expression of mitochondrial ETC components during *in vivo* growth compared to *in vitro* culture (Daily et al., 2007; van Dooren and McFadden, 2007).

This study has described a new class of highly selective *P. falciparum* inhibitors predicted to target the Q_o site of the bc_1 complex. The ability of these compounds to additionally disrupt hemozoin formation makes them attractive inhibitors which merit further drug development. This view is strengthened by the potent pM antimalarial activity displayed by the recently synthesized haloalkoxyacridones (Winter et al., 2006). Furthermore, we predict that by assessing the inhibitory activity of these molecules against human bc_1, it may be possible to circumvent toxicological issues previously encountered during the development of other dihydroacridinediones such as floxacrine (Raether and Fink, 1982).

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This article has not been copyedited and formatted. The final version may differ from this version.

References

Allen RJ and Kirk K (2004) The membrane potential of the intraerythrocytic malaria parasite Plasmodium falciparum. *J Biol Chem* **279**(12):11264-11272.

Auparakkitanon S, Chapoomram S, Kuaha K, Chirachariyavej T and Wilairat P (2006) Targeting of hematin by the antimalarial pyronaridine. *Antimicrob Agents Chemother* **50**(6):2197-2200.

Auparakkitanon S, Noonpakdee W, Ralph RK, Denny WA and Wilairat P (2003) Antimalarial 9-anilinoacridine compounds directed at hematin. *Antimicrob Agents Chemother* **47**(12):3708-3712.

Auparakkitanon S and Wilairat P (2000) Cleavage of DNA induced by 9-anilinoacridine inhibitors of topoisomerase II in the malaria parasite Plasmodium falciparum. *Biochem Biophys Res Commun* **269**(2):406-409.

Basco LK, Mitaku S, Skaltsounis AL, Ravelomanantsoa N, Tillequin F, Koch M and Le Bras J (1994) In vitro activities of furoquinoline and acridone alkaloids against Plasmodium falciparum. *Antimicrob Agents Chemother* **38**(5):1169-1171.

Berenbaum MC (1978) A method for testing for synergy with any number of agents. *J Infect Dis* **137**(2):122-130.

Biagini GA, O'Neill PM, Bray PG and Ward SA (2005) Current drug development portfolio for antimalarial therapies. *Curr Opin Pharmacol* **5**(5):473-478.

Biagini GA, O'Neill PM, Nzila A, Ward SA and Bray PG (2003) Antimalarial chemotherapy: young guns or back to the future? *Trends Parasitol* **19**(11):479-487.

Biagini GA, Viriyavejakul P, O'Neill P M, Bray PG and Ward SA (2006) Functional characterization and target validation of alternative complex I of Plasmodium falciparum mitochondria. *Antimicrob Agents Chemother* **50**(5):1841-1851.

Bray PG, Janneh O, Raynes KJ, Mungthin M, Ginsburg H and Ward SA (1999) Cellular uptake of chloroquine is dependent on binding to ferriprotoporphyrin IX and is independent of NHE activity in Plasmodium falciparum. *J Cell Biol* **145**(2):363-376.

Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol* **22**(23):3099-3108.

Chou AC and Fitch CD (1993) Control of heme polymerase by chloroquine and other quinoline derivatives. *Biochem Biophys Res Commun* **195**(1):422-427.

Crofts AR (2004) The cytochrome bc1 complex: function in the context of structure. *Annu Rev Physiol* **66**:689-733.

Crofts AR, Barquera B, Gennis RB, Kuras R, Guergova-Kuras M and Berry EA (1999) Mechanism of ubiquinol oxidation by the bc(1) complex: different domains of the quinol binding pocket and their role in the mechanism and binding of inhibitors. *Biochemistry* **38**(48):15807-15826.

Daily JP, Scanfeld D, Pochet N, Le Roch K, Plouffe D, Kamal M, Sarr O, Mboup S, Ndir O, Wypij D, Levasseur K, Thomas E, Tamayo P, Dong C, Zhou Y, Lander ES, Ndiaye D, Wirth D, Winzeler EA, Mesirov JP and Regev A (2007) Distinct physiological states of Plasmodium falciparum in malaria-infected patients. *Nature*.

Desjardins RE, Canfield CJ, Haynes JD and Chulay JD (1979) Quantitative assessment of antimalarial activity in vitro by a semiautomated microdilution technique. *Antimicrob Agents Chemother* **16**(6):710-718.

Dorn A, Scovill JP, Ellis WY, Matile H, Ridley RG and Vennerstrom JL (2001) Short report: floxacrine analog WR 243251 inhibits hematin polymerization. *Am J Trop Med Hyg* **65**(1):19-20.

Dorn A, Vippagunta SR, Matile H, Jaquet C, Vennerstrom JL and Ridley RG (1998) An assessment of drug-haematin binding as a mechanism for inhibition of haematin polymerisation by quinoline antimalarials. *Biochem Pharmacol* **55**(6):727-736.

Durckheimer W, Raether W, Seliger H and Seidenath H (1980) 10-Hydroxy-3,4-dihydroacridine-1,9(2H,10H)-diones, a new group of malaricidal and coccidiostatic compounds. *Arzneimittelforschung* **30**(7):1041-1046.

Edwards G and Biagini GA (2006) Resisting resistance: dealing with the irrepressible problem of malaria. *Br J Clin Pharmacol* **61**(6):690-693.

Egan TJ, Mavuso WW, Ross DC and Marques HM (1997) Thermodynamic factors controlling the interaction of quinoline antimalarial drugs with ferriprotoporphyrin IX. *J Inorg Biochem* **68**(2):137-145.

Esser L, Quinn B, Li YF, Zhang M, Elberry M, Yu L, Yu CA and Xia D (2004) Crystallographic studies of quinol oxidation site inhibitors: a modified classification of inhibitors for the cytochrome bc(1) complex. *J Mol Biol* **341**(1):281-302.

Fisher N, Bray PG, Ward SA and Biagini GA (2008) Malaria-parasite mitochondrial dehydrogenases as drug targets: too early to write the obituary. *Trends Parasitol* **24**(1):9 - 10.

Fisher N, Brown AC, Sexton G, Cook A, Windass J and Meunier B (2004a) Modeling the Qo site of crop pathogens in Saccharomyces cerevisiae cytochrome b. *Eur J Biochem* **271**(11):2264-2271.

Fisher N, Castleden CK, Bourges I, Brasseur G, Dujardin G and Meunier B (2004b) Human disease-related mutations in cytochrome b studied in yeast. *J Biol Chem* **279**(13):12951-12958.

Fisher N and Meunier B (2005) Re-examination of inhibitor resistance conferred by Qo-site mutations in cytochrome b using yeast as a model system. *Pest Manag Sci* **61**(10):973-978.

Fry M and Pudney M (1992) Site of action of the antimalarial hydroxynaphthoquinone, 2-[trans-4- (4'-chlorophenyl) cyclohexyl]-3-hydroxy-1,4-naphthoquinone (566C80). *Biochem Pharmacol* **43**(7):1545-1553.

Gamage SA, Tepsiri N, Wilairat P, Wojcik SJ, Figgitt DP, Ralph RK and Denny WA (1994) Synthesis and in vitro evaluation of 9-anilino-3,6-diaminoacridines active against a multidrug-resistant strain of the malaria parasite Plasmodium falciparum. *J Med Chem* **37**(10):1486-1494.

Gazarini ML and Garcia CR (2004) The malaria parasite mitochondrion senses cytosolic Ca2+ fluctuations. *Biochem Biophys Res Commun* **321**(1):138-144.

Gill HJ, Tingle MD and Park BK (1995) N-Hydroxylation of dapsone by multiple enzymes of cytochrome P450: implications for inhibition of haemotoxicity. *Br J Clin Pharmacol* **40**(6):531-538.

Goodman CD, Su V and McFadden GI (2007) The effects of anti-bacterials on the malaria parasite Plasmodium falciparum. *Mol Biochem Parasitol* **152**(2):181-191.

Gutteridge WE, Dave D and Richards WH (1979) Conversion of dihydroorotate to orotate in parasitic protozoa. *Biochim Biophys Acta* **582**(3):390-401.

Hammond DJ, Burchell JR and Pudney M (1985) Inhibition of pyrimidine biosynthesis de novo in Plasmodium falciparum by 2-(4-t-butylcyclohexyl)-3-hydroxy-1,4-naphthoquinone in vitro. *Mol Biochem Parasitol* **14**(1):97-109.

Hetenyi C and van der Spoel D (2002) Efficient docking of peptides to proteins without prior knowledge of the binding site. *Protein Sci* **11**(7):1729-1737.

Kesten SJ, Degnan MJ, Hung J, McNamara DJ, Ortwine DF, Uhlendorf SE and Werbel LM (1992) Synthesis and antimalarial properties of 1-imino derivatives of 7-chloro-3-substituted-3,4-dihydro-1,9(2H,10H)-acridinediones and related structures. *J Med Chem* **35**(19):3429-3447.

Kobayashi T, Sato S, Takamiya S, Komaki-Yasuda K, Yano K, Hirata A, Onitsuka I, Hata M, Mi-Ichi F, Tanaka T, Hase T, Miyajima A, Kawazu S, Watanabe Y and Kita K (2007) Mitochondria and

apicoplast of Plasmodium falciparum: Behaviour on subcellular fractionation and the implication. *Mitochondrion* **7**(1-2):125-132.

Korsinczky M, Chen N, Kotecka B, Saul A, Rieckmann K and Cheng Q (2000) Mutations in Plasmodium falciparum cytochrome b that are associated with atovaquone resistance are located at a putative drug-binding site. *Antimicrob Agents Chemother* **44**(8):2100-2108.

Kuboyama M, Yong FC and King TE (1972) Studies on cytochrome oxidase. 8. Preparation and some properties of cardiac cytochrome oxidase. *J Biol Chem* **247**(20):6375-6383.

Lambros C and Vanderberg JP (1979) Synchronization of Plasmodium falciparum erythrocytic stages in culture. *J Parasitol* **65**(3):418-420.

Margoliash E and Walasek OF (eds) (1967) *Cytochrome c from vertebrate and invertebrate sources.*

Marques HM, Voster K and Egan TJ (1996) The interaction of the heme-octapeptide, N-acetylmicroperoxidase-8 with antimalarial drugs: solution studies and modeling by molecular mechanics methods. *J Inorg Biochem* **64**(1):7-23.

Mitchell P (1975) The protonmotive Q cycle: a general formulation. *FEBS Lett* **59**(2):137-139.

Morris GM, Goodsell DS, Halliday RS, Huey R, Hart WE, Belew RK and Olson AJ (1998) *Automated docking using a Lamarckian genetic algorithm and empirical binding free energy function*. *J Computational Chemistry* **19**:1639-1662.

Oettmeier W, Masson K and Kalinna S (1995) [3H]7-azido-4-isopropylacridone labels Cys159 of the bovine mitochondrial ADP/ATP-carrier protein. *Eur J Biochem* **227**(3):730-733.

Oettmeier W, Masson K and Soll M (1992) The acridones, new inhibitors of mitochondrial NADH: ubiquinone oxidoreductase (complex I). *Biochim Biophys Acta* **1099**(3):262-266.

Oettmeier W, Masson K, Soll M and Reil E (1994) Acridones and quinolones as inhibitors of ubiquinone functions in the mitochondrial respiratory chain. *Biochem Soc Trans* **22**(1):213-216.

Painter HJ, Morrisey JM, Mather MW and Vaidya AB (2007) Specific role of mitochondrial electron transport in blood-stage Plasmodium falciparum. *Nature* **446**(7131):88-91.

Raether W and Fink E (1979) Antimalarial activity of Floxacrine (HOE 991) I. Studies on blood schizontocidal action of Floxacrine against Plasmodium berghei, P. vinckei and P. cynomolgi. *Ann Trop Med Parasitol* **73**(6):505-526.

Raether W and Fink E (1982) Antimalarial activity of floxacrine (HOE 991) II: Studies on causal prophylactic and blood schizontocidal action of floxacrine and related dihydroacridinediones against Plasmodium yoelii and P. berghei. *Ann Trop Med Parasitol* **76**(5):507-516.

Schmidt LH (1979) Antimalarial properties of floxacrine, a dihydroacridinedione derivative. *Antimicrob Agents Chemother* **16**(4):475-485.

Shao BR (1990) A review of antimalarial drug pyronaridine. *Chin Med J (Engl)* **103**(5):428-434.

Snow RW, Trape JF and Marsh K (2001) The past, present and future of childhood malaria mortality in Africa. *Trends Parasitol* **17**(12):593-597.

Srivastava IK, Morrisey JM, Darrouzet E, Daldal F and Vaidya AB (1999) Resistance mutations reveal the atovaquone-binding domain of cytochrome b in malaria parasites. *Mol Microbiol* **33**(4):704-711.

Stead AM, Bray PG, Edwards IG, DeKoning HP, Elford BC, Stocks PA and Ward SA (2001) Diamidine compounds: selective uptake and targeting in Plasmodium falciparum. *Mol Pharmacol* **59**(5):1298-1306.

Suswam E, Kyle D and Lang-Unnasch N (2001) Plasmodium falciparum: The Effects of Atovaquone Resistance on Respiration. *Exp Parasitol* **98**(4):180-187.

Syafruddin D, Siregar JE and Marzuki S (1999) Mutations in the cytochrome b gene of Plasmodium berghei conferring resistance to atovaquone [In Process Citation]. *Mol Biochem Parasitol* **104**(2):185-194.

Trager W and Jensen JB (1976) Human malaria parasites in continuous culture. *Science* **193**(4254):673-675.

Uyemura SA, Luo S, Moreno SN and Docampo R (2000) Oxidative phosphorylation, Ca(2+) transport, and fatty acid-induced uncoupling in malaria parasites mitochondria. *J Biol Chem* **275**(13):9709-9715.

van Dooren GG and McFadden GI (2007) Malaria: differential parasite drive. *Nature* **450**(7172):955-956.

Vanneste WH (1966) Molecular proportion of the fixed cytochrome components of the respiratory chain of Keilin-Hartree particles and beef heart mitochondria. *Biochim Biophys Acta* **113**(1):175-178.

Wernsdorfer WH and Payne D (1991) The dynamics of drug resistance in Plasmodium falciparum. *Pharmacol Ther* **50**(1):95-121.

Winter RW, Kelly JX, Smilkstein MJ, Dodean R, Bagby GC, Rathbun RK, Levin JI, Hinrichs D and Riscoe MK (2006) Evaluation and lead optimization of anti-malarial acridones. *Exp Parasitol* **114**(1):47-56.

Footnote

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Legends for figures

Figure 1. Chemical structure of key bc_1 inhibitors used in this study.

Figure 2. Ubiquinol (QH_2) concentration dependent cytochrome *c* reduction of (a) *P. falciparum* cell-free extracts and (b) human liver microsomes. Data points are means from observations from three individual experiments. Data were fitted to a function describing simple ligand binding at a single site by nonlinear regression analysis (Marquart method) using an iterative procedure to generate the best fit ($\chi 2$) of the curve to the data.

Figure 3. Effect of WR249685 on mitochondrial $\Delta\Psi_m$. Time course of TMRE-dependent fluorescence after the addition of WR249685 (10 μM) to (a) untreated and (b) bafilomycin A_1 (200 nM) treated *P. falciparum*-infected erythrocytes. Data were normalized to 100% in (a) untreated or (b) bafilomycin-treated cells and to 0% in FCCP (10 μM)-treated cells. Graphs show means from experiments performed independently ± standard errors ($n \geq 3$).

Figure 4. Isobole analysis of the fractional inhibitory concentrations (FIC) of IC_{50} values for atovaquone versus WR249685 (figure is representative of 3 independent experiments).

Figure 5. (a) Sequence alignment of Q_o site regions from bovine (Bt), human (Hs), *P. falciparum* (Pf), rat (Rn) and Baker's yeast (Sc) cytochrome *b*. Helices are identified by arrows above the alignment. Open squares below the sequence data identify residues in close contact (< 3.5Å separation, hydrogen bonding or 40Å2 surface contact) with stigmatellin in 1SQX.pdb(Esser et al., 2004) ; filled circles identify residues in

close contact with myxothiazol in 1SQP.pdb (ibid), and asterisks identify residues associated with atovaquone resistance mutations in *Plasmodium* sp, *Toxoplasma gondii* and *Pneumocystis jirovecii*. The grey shaded area identifies residues located within a 4Å radius of WR249685 docked into the *P. falciparum* homology model of cytochrome *b*. (b) Structural alignment of selected Q_o site regions from bovine cytochrome *b* (1SQX.pdb, dark grey) and the homology model of *P. falciparum* cytochrome *b* (light grey). Highlighted residues are discussed in the text.

Figure 6. (a) SWISS-PROT homology model of *P.falciparum* cytochrome *b* in cartoon representation showing WR249685 docked at the Qo site region. The protein backbone is represented in orange, with the hemes in red wireframe. WR249685 and associated liganding residues are represented in white and green wireframe respectively (b) Detail of WR249685 (white wireframe) docked at Q_o in the *P. falciparum* homology model showing residues within a 4Å radius of the bound inhibitor (CPK wireframe).

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Table 1. Inhibition profiles of bovine, rat, human and *P. falciparum* bc_1 activity[a].

Inhibitor	*Bovine heart bc_1 inhibition IC_{50} ± SEM (nM)[b]*	*Rat liver bc_1 inhibition IC_{50} ± SEM (nM)[c]*	*Human liver bc_1 inhibition IC_{50} ± SEM (nM)[d]*	*K_i human liver bc_1 (nM)[e]*	*P.falciparum bc_1 inhibition IC_{50} ± SEM (nM)*	*K_i P. falciparum bc_1 (nM)[e]*	*In vitro therapeutic index[f]*	*P. falciparum growth inhibition IC_{50} ± SEM (nM)*
Stigmatellin	2.4	9 ± 1	15 ± 0.2	2	12 ± 1	1.3	1	N.D.
Myxothiazol	8.4	16 ± 2	15 ± 1	2	3.5 ± 0.5	0.4	4	N.D.
Atovaquone	83± 23	406 ± 30	72 ± 9	10	3 ± 2	0.3	24	1 ± 0.2
Pyridone (GW844520)	51± 9	353 ± 47	153 ± 16	21	32 ± 13	3.5	5	15 ± 2
WR 249685	>13800	>13800	>13800	N	3 ± 2	0.3	>4600	15 ± 6
Floxacrine (racemic)	328 ± 65	1205 ± 340	1458 ± 280	198	803 ± 183	89	2	140 ± 33

[a] bc_1 activity was determined by monitoring cytochrome *c* reduction using decylubiquinol as electron donor (see methods). All data acquired from multiple observations from at least two separate preparations. DMSO in the assays did not exceed 0.3 %. IC_{50}s were calculated by using the four-parameter logistic method.
[b] Assays contained 30 nM cyt *b.*
[c] Assays contained 7 *nM cyt b.*
[d] Assays contained 30 *nM cyt b.*
[e] Inhibitory equilibrium dissociation constants (K_i) for antagonists were determined using the Cheng-Prusoff equation (see methods).
[f] Calculated by dividing human bc_1 IC_{50} values by *P.falciparum* bc_1 IC_{50} values.
N, Not determinable.
N.D., Not Done.

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Table 2 Effect of bc_1 Q_o mutations on inhibition profiles in yeast[a].

Inhibitor	*Yeast WT bc_1 inhibition IC_{50} ± SEM (nM)[b]*	*Y279S bc_1 inhibition IC_{50} ± SEM (nM)[c]*	*G143A bc_1 inhibition IC_{50} ± SEM (nM)[d]*
Atovaquone	3.5 ± 3	2689 ± 462	27 ± 3
Pyridone (GW844520)	189 ± 31	3217 ± 268	212 ± 42
WR 249685	32% inhibition at 2780 40% inhibition at 5600	No inhibition at 5600	5 % inhibition at 5600
Floxacrine (racemic)	8446 ± 5943	>28000	12599 ± 2543

[a] bc_1 activity was determined by monitoring cytochrome *c* reduction using decylubiquinol as electron donor (see methods). All assays contained 5 nM cyt *b*. DMSO in the assays did not exceed 0.3 %. All data acquired from multiple observations from at least two separate preparations. IC_{50}s were calculated by using the four-parameter logistic method.

Atovaquone

Pryridone (GW844520)

WR 249685
(*S* enantiomer)

Floxacrine
(racemic)

Fig 1

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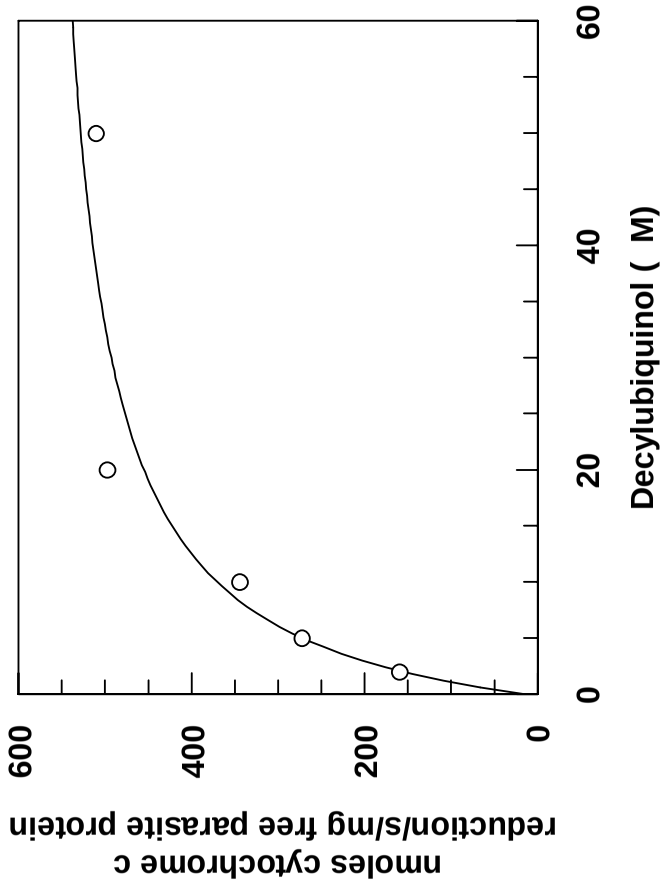

Fig. 2A

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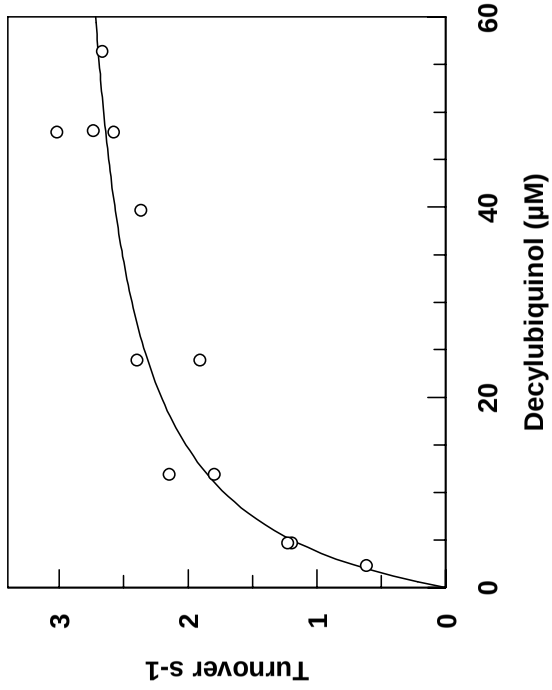

Fig. 2B

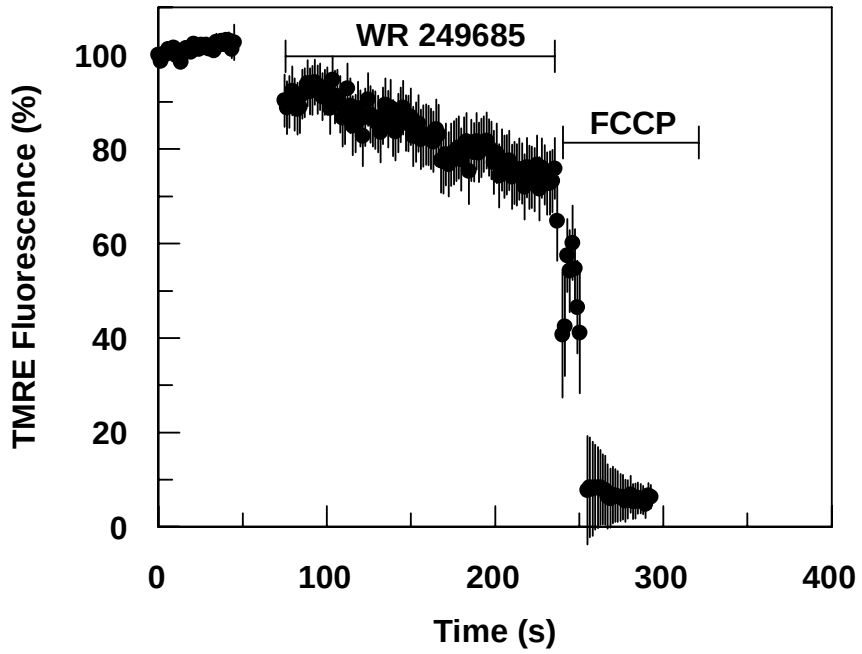

Fig. 3A

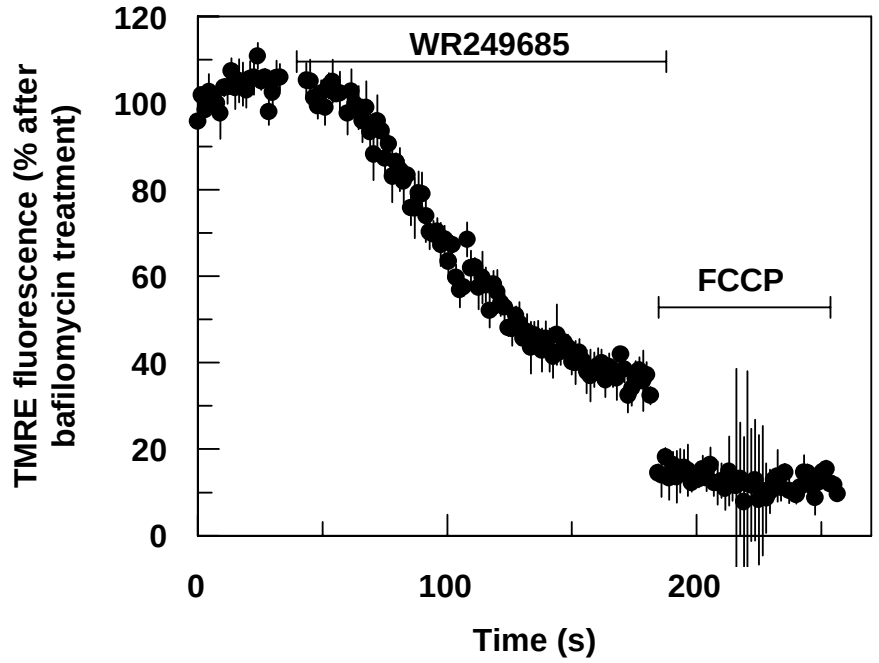

Fig. 3B

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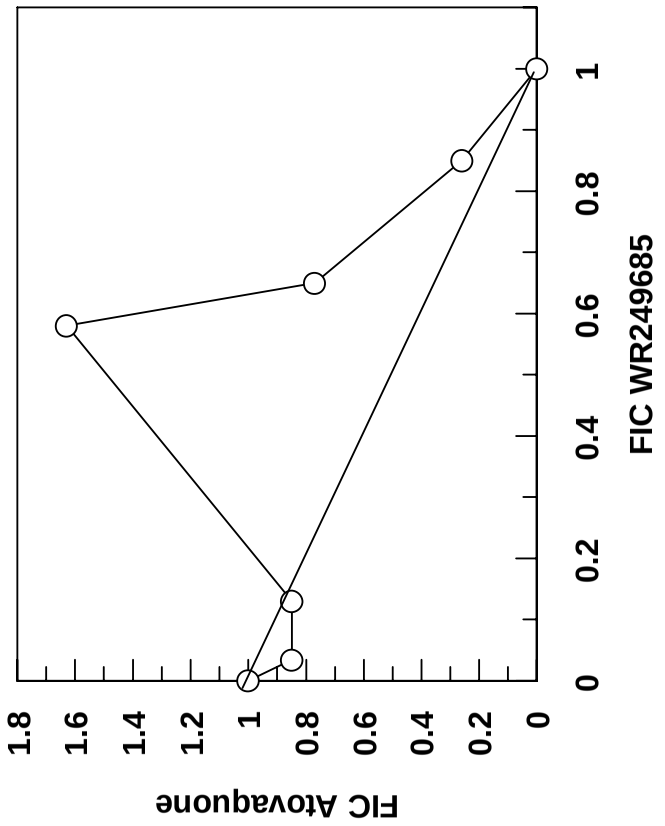

Fig. 4

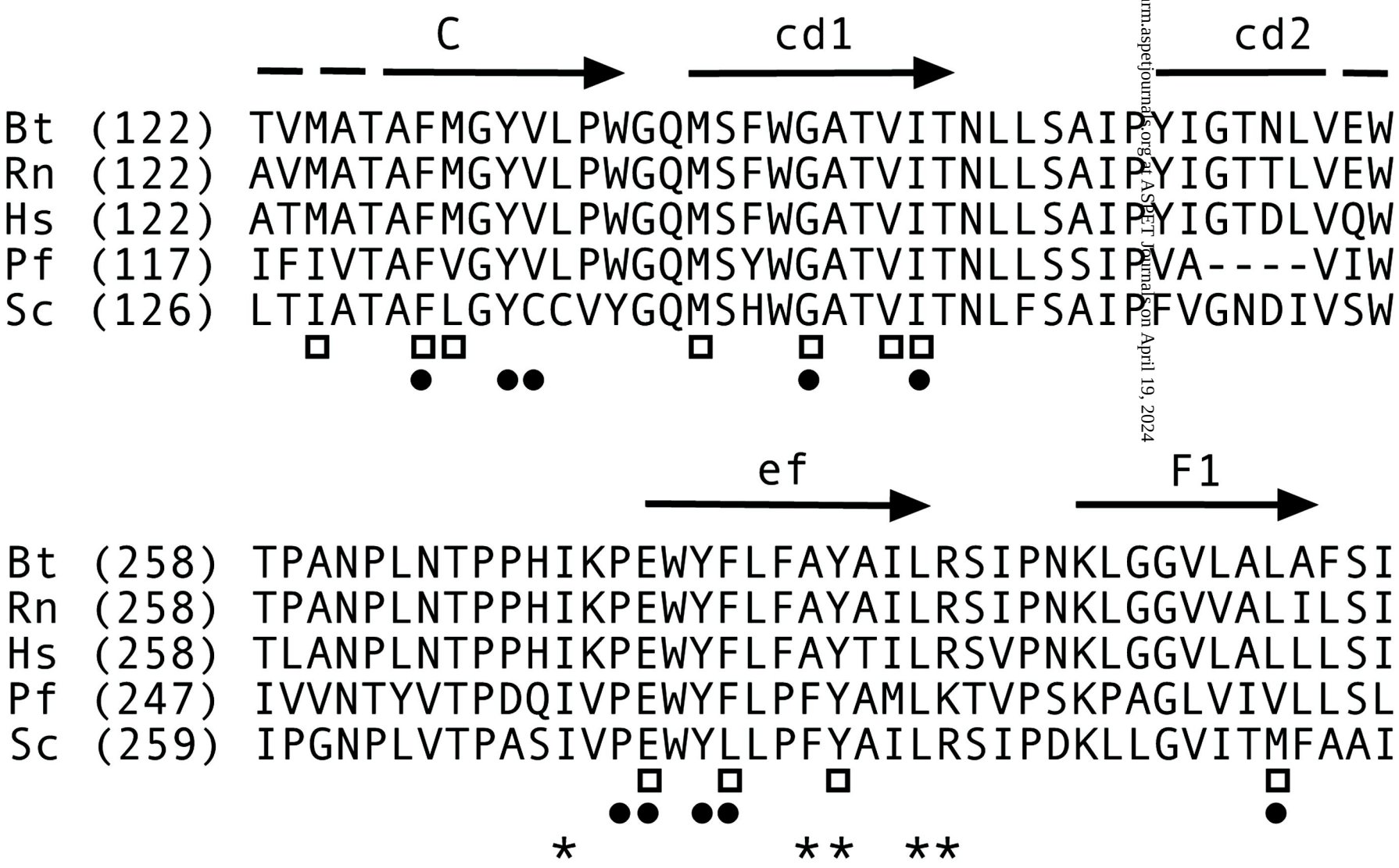

Fig. 5A

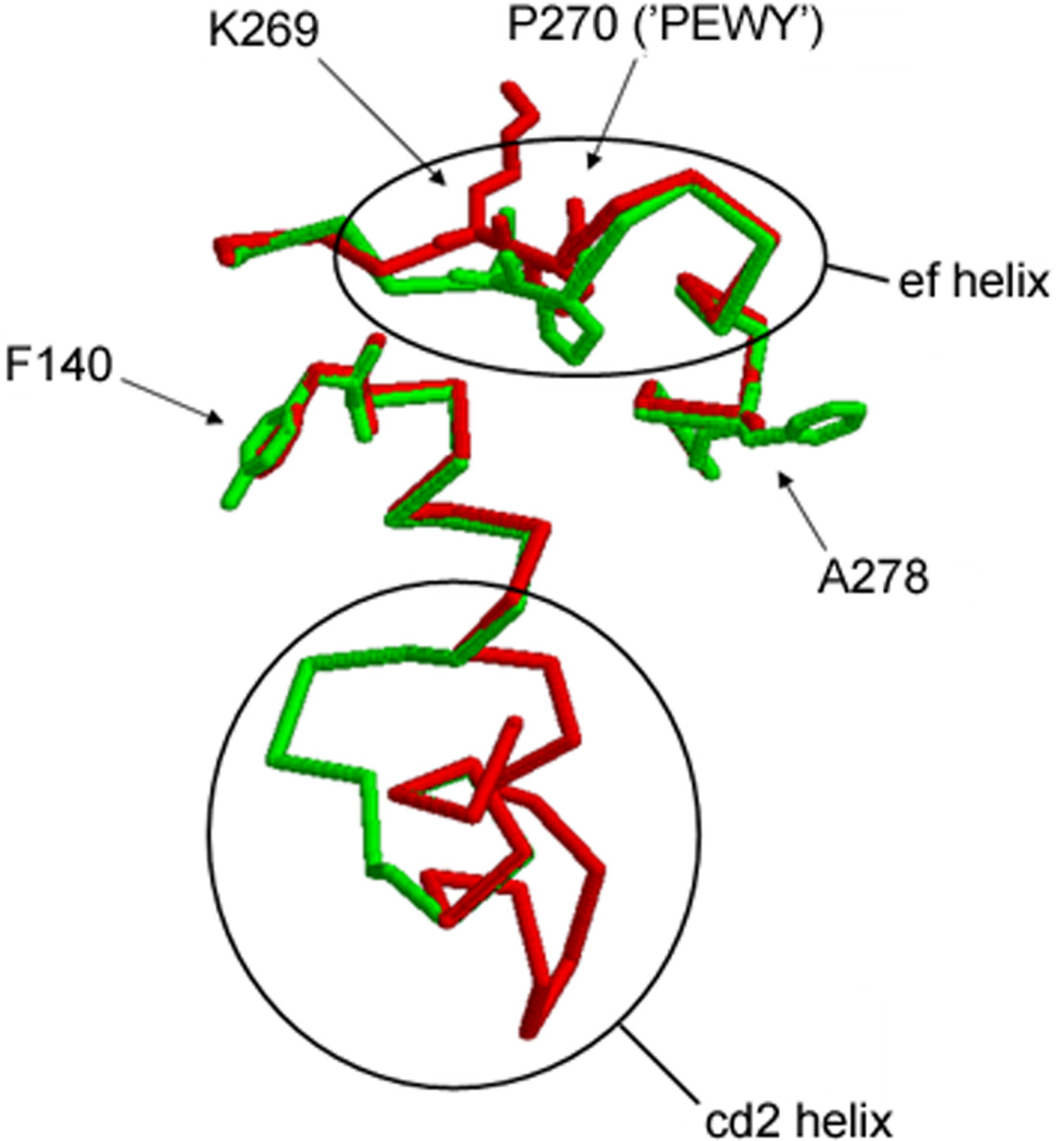

Fig. 5B

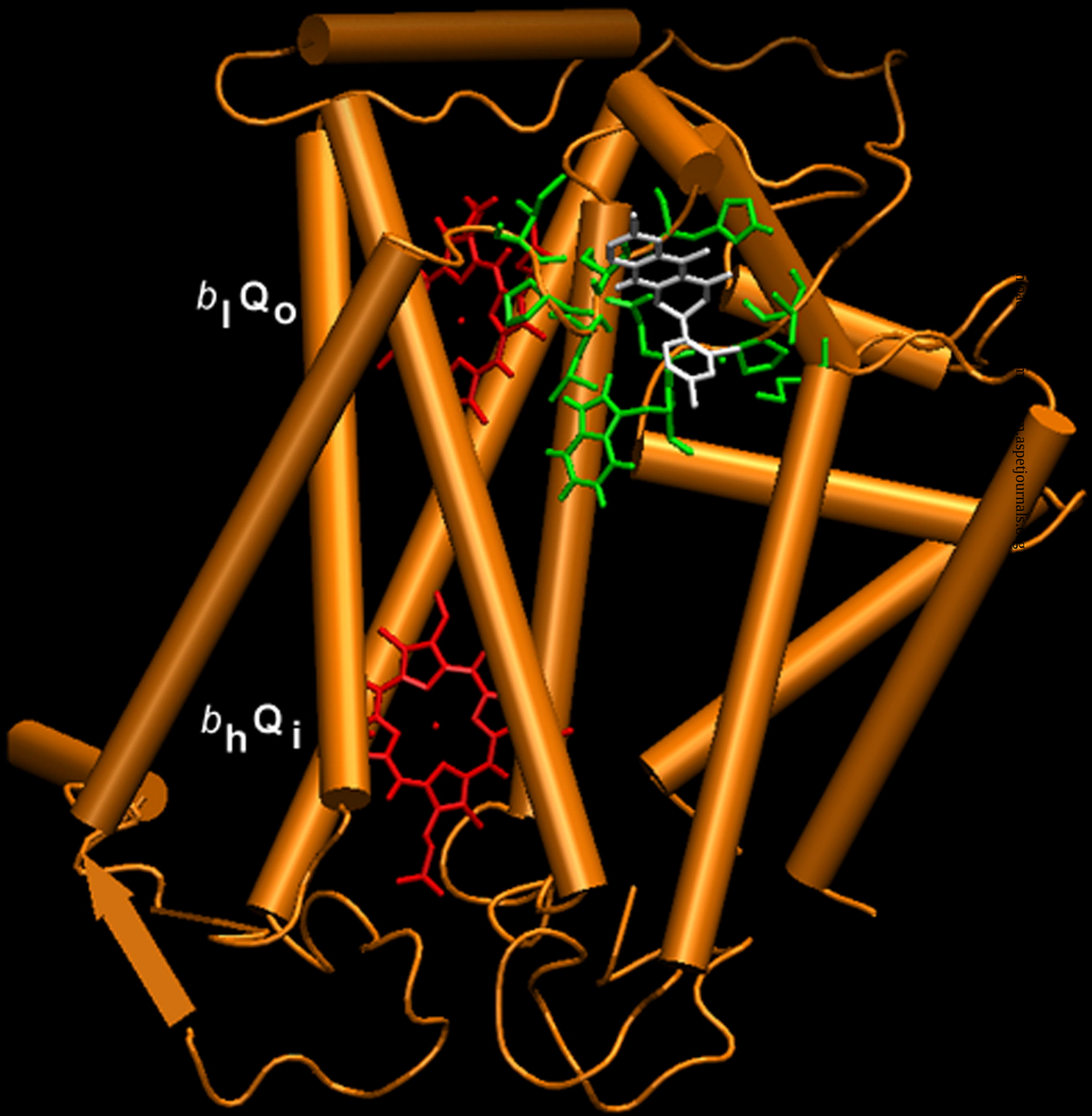

Fig. 6A

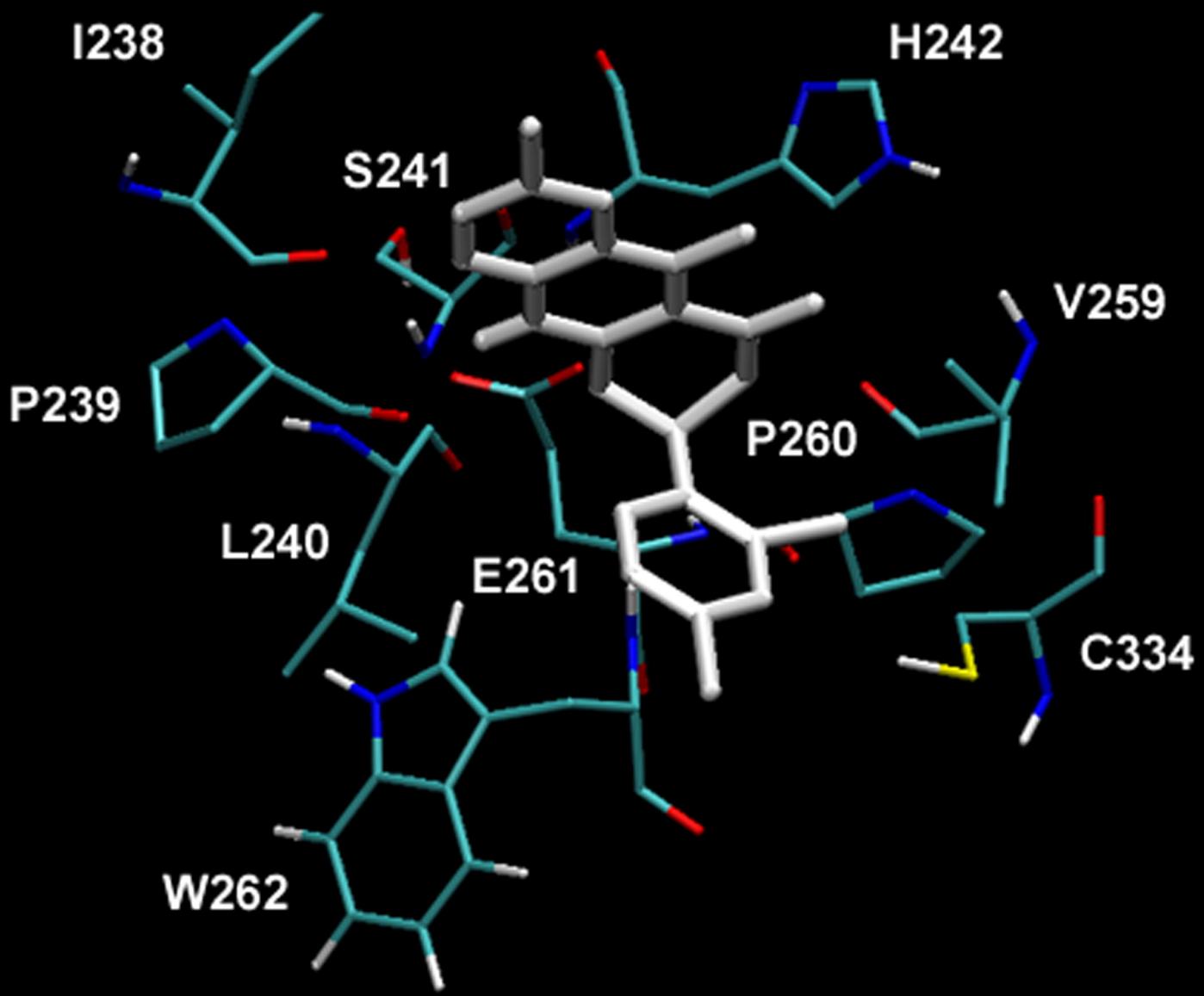

Fig. 6B