

ACRIDINEDIONES: SELECTIVE AND POTENT INHIBITORS OF THE MALARIA PARASITE MITOCHONDRIAL *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.

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 ($IC_{50} \sim 15nM$). 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).

Whilst some dihydroacridinediones have been reported to inhibit the malaria parasite respiratory pathway, causing a reduction in whole cell O₂ 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.

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 NaHCO₃) 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% O₂, 3% CO₂ in N₂ 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 [³H]-hypoxanthine incorporation method (Desjardins et al., 1979) with an inoculum size of 0.5 % parasitemia (ring stage) and 1% hematocrit. IC₅₀s 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 IC₅₀s. The fractional inhibitory concentrations of the resulting IC₅₀s 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

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 μ M) 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.

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

hexane under N₂. 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 $\epsilon_{288-320} = 4.14 \text{ mM}^{-1} \text{ cm}^{-1}$.

Measurement of bc₁ 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 $\epsilon_{562-575} = 28.5 \text{ mM}^{-1} \text{ cm}^{-1}$ (Vanneste, 1966). Turnover rates of cytochrome *c* reduction were determined using $\epsilon_{550-542} = 18.1 \text{ mM}^{-1} \text{ cm}^{-1}$ (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). IC₅₀s 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 ($\Delta\Psi_m$) 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

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 Biopetechs 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

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.

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 μ M, respectively. WR249685 inhibited hemozoin formation by 50 % at 130 μ M, 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 μ M for CQ, 1.55 μ M for AQ, 1.87 μ M for floxacrine and 31.74 μ M 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 bc₁ 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 (QH₂) as electron donor.

P. falciparum bc₁ activity exhibited Michaelis-Menten kinetics with an apparent concentration of substrate leading to half-maximal velocity (K_m) for QH₂ of 6.2 ± 2 μ M reaching a maximum/limiting velocity (V_{max}) of

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 QH₂ 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.

Inhibition of yeast bc₁ 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 bc₁ inhibitory activity against wild type yeast (IC₅₀ 3 nM) and G143A mutants (IC₅₀ 27 nM), whilst the Y279S mutation conferred significant resistance (IC₅₀ 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 bc₁ complex.

Dihydroacridinediones collapse mitochondrial membrane potential ΔΨ_m. The measurement of mitochondrial ΔΨ_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 ΔΨ_m. This phenomenon has been observed previously (Biagini et al., 2006) and is the result of the high ΔΨ_m (–100 mV) of the plasma membrane (Allen and Kirk, 2004). Upon addition of the V-type H⁺ ATPase inhibitors bafilomycin A₁ or concanamycin (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 ΔΨ_m contribute to the accumulation of TMRE, we could not accurately quantify the finite ΔΨ_m values. Therefore, for all experiments, the fluorescence dynamic range was set up so that untreated TMRE-loaded cells were regarded as having complete

fluorescence (100%), while the baseline (0%) was set by addition of the protonophore FCCP (10 μ M). For mitochondrial-dependent fluorescence, bafilomycin A₁-treated cells were normalized to 100% and, again, the baseline (0%) was set by FCCP (10 μ M).

Addition of the dihydroacridinediones, WR249685 (Fig. 3a) and floxacrine (not shown), was observed to partially reduce (~20%) the total cellular $\Delta\Psi_m$ -dependent TMRE fluorescence, possibly indicating an effect on the mitochondrial contribution. This view was confirmed by the reduction of mitochondrial $\Delta\Psi_m$ -dependent fluorescence from bafilomycin A₁-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 IC₅₀'s. The resultant isobologram of atovaquone and WR249685 is suggestive of a possible antagonistic interaction (Fig. 4).

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 IC₅₀ 140 nM, compared to CQ IC₅₀ 7.4 nM and AQ IC₅₀ 4.5 nM. However, WR249685 demonstrated potent *in vitro* antimalarial activity (IC₅₀ 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

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 binding 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

inhibitor (Table 2). So what is it about the *P. falciparum* bc₁ 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 mammalian 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* bc₁ 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

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 IC₅₀ for atovaquone in rat liver microsomes 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 potential 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

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).

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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Footnote

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Legends for figures

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

Figure 2. Ubiquinol (QH₂) 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 (χ^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 μ M) to (a) untreated and (b) bafilomycin A₁ (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 μ M)-treated cells. Graphs show means from experiments performed independently \pm standard errors ($n \geq 3$).

Figure 4. Isobole analysis of the fractional inhibitory concentrations (FIC) of IC₅₀ 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 ($< 3.5\text{\AA}$ separation, hydrogen bonding or 40\AA^2 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 Q_o 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).

Table 1. Inhibition profiles of bovine, rat, human and *P. falciparum* bc₁ activity^a.

<i>Inhibitor</i>	<i>Bovine heart bc₁ inhibition IC₅₀ ± SEM (nM)^b</i>	<i>Rat liver bc₁ inhibition IC₅₀ ± SEM (nM)^c</i>	<i>Human liver bc₁ inhibition IC₅₀ ± SEM (nM)^d</i>	<i>K_i human liver bc₁ (nM)^e</i>	<i>P.falciparum bc₁ inhibition IC₅₀ ± SEM (nM)</i>	<i>K_i P. falciparum bc₁ (nM)^e</i>	<i>In vitro therapeutic index^f</i>	<i>P. falciparum growth inhibition IC₅₀ ± SEM (nM)</i>
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

^abc₁ 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 %. IC₅₀s 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 bc₁ IC₅₀ values by *P.falciparum* bc₁ IC₅₀ values.

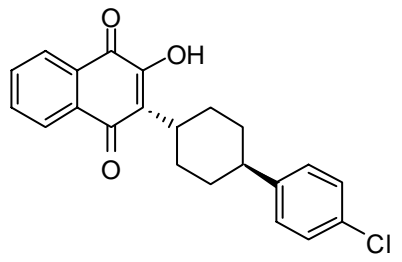
N, Not determinable.

N.D., Not Done.

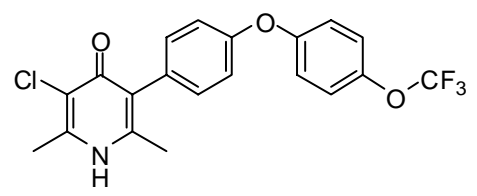
Table 2 Effect of bc_1 Q_o mutations on inhibition profiles in yeast^a.

<i>Inhibitor</i>	<i>Yeast WT bc_1 inhibition</i> <i>IC₅₀ ± SEM (nM)^b</i>	<i>Y279S bc_1 inhibition</i> <i>IC₅₀ ± SEM (nM)^c</i>	<i>G143A bc_1 inhibition</i> <i>IC₅₀ ± SEM (nM)^d</i>
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

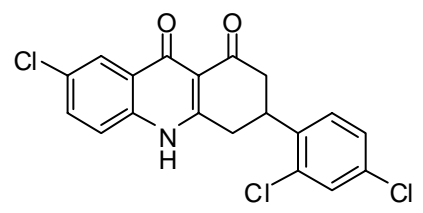
^a bc_1 activity was determined by monitoring cytochrome *c* reduction using decylubiquinol as electron donor (see methods). All assays contained 5 nM cyt *b*. DMSO in the assays did not exceed 0.3 %. All data acquired from multiple observations from at least two separate preparations. IC₅₀s were calculated by using the four-parameter logistic method.



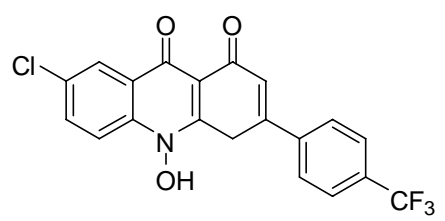
Atovaquone



Pryridone (GW844520)



WR 249685
(S enantiomer)



Floxacin
(racemic)

Fig 1

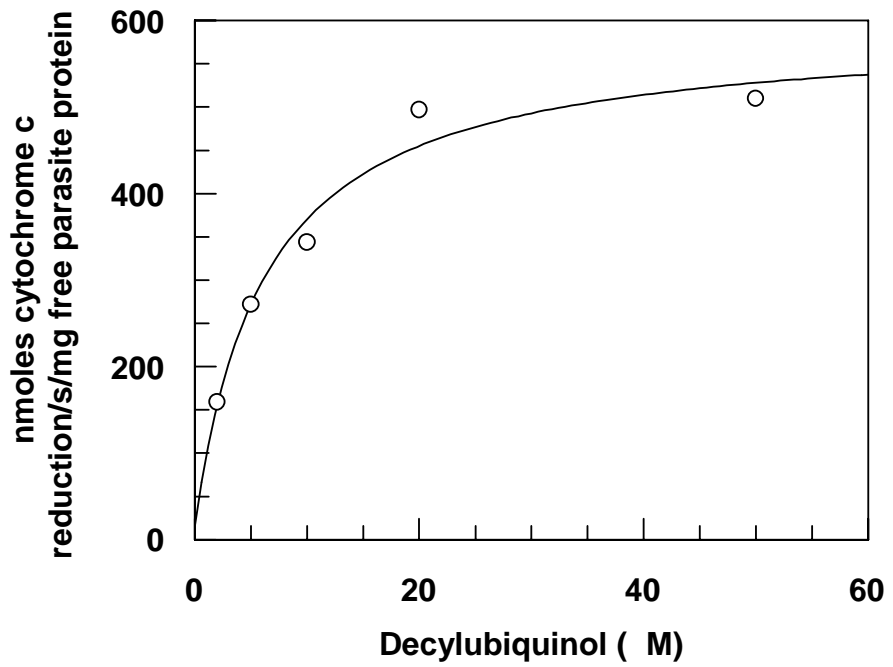


Fig. 2A

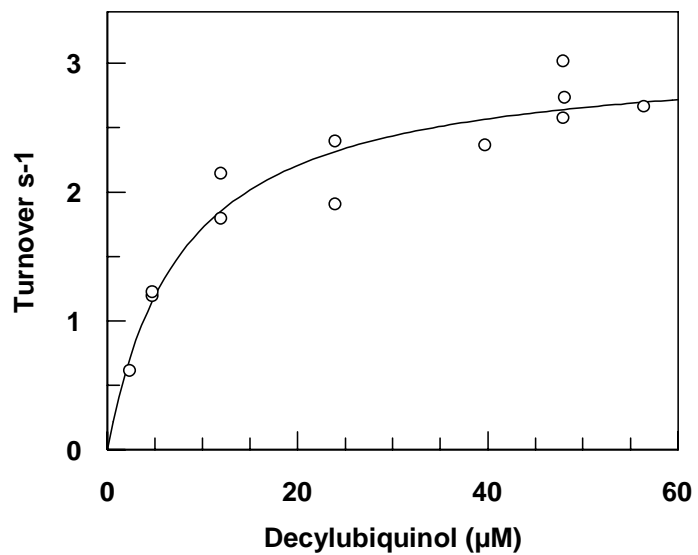


Fig. 2B

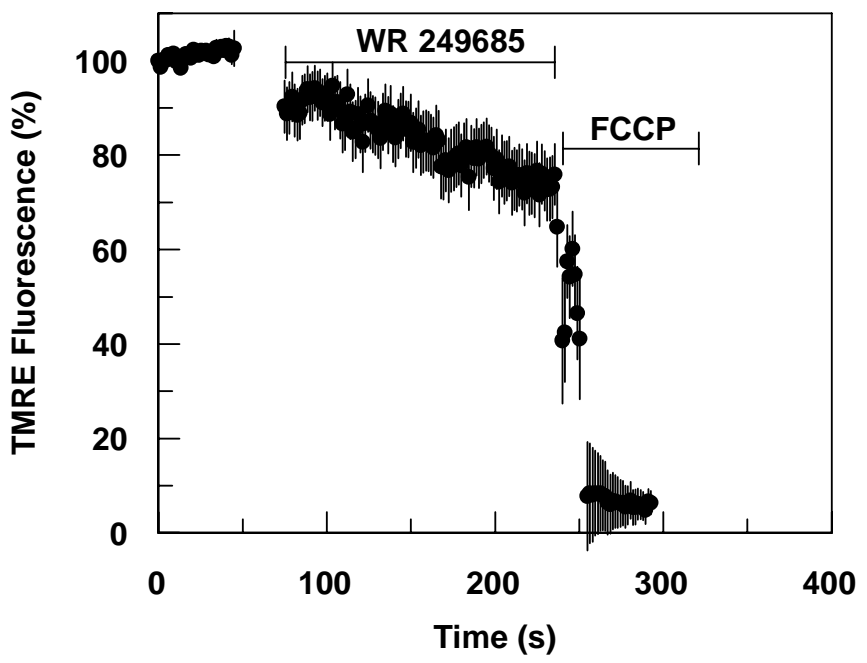


Fig. 3A

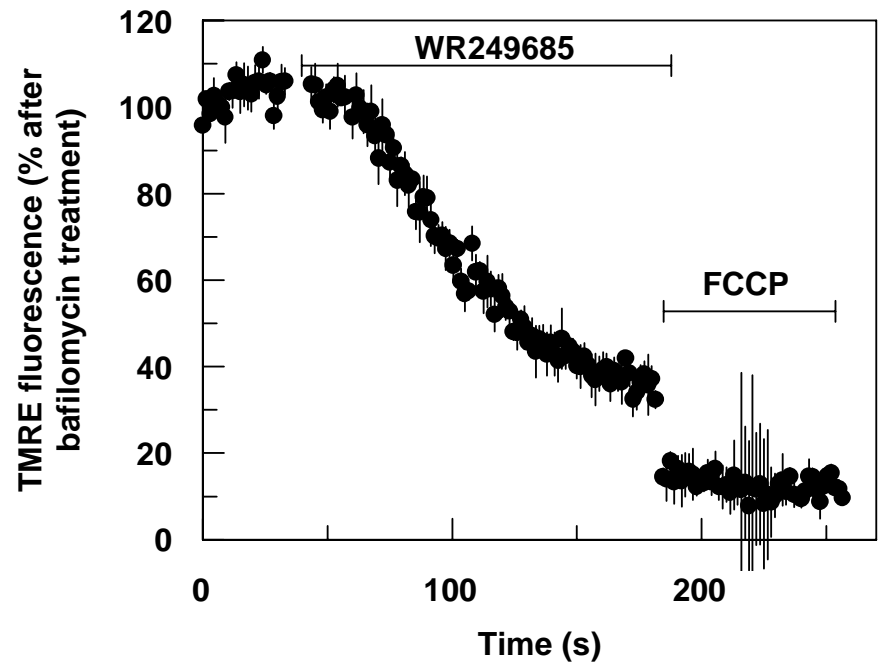


Fig. 3B

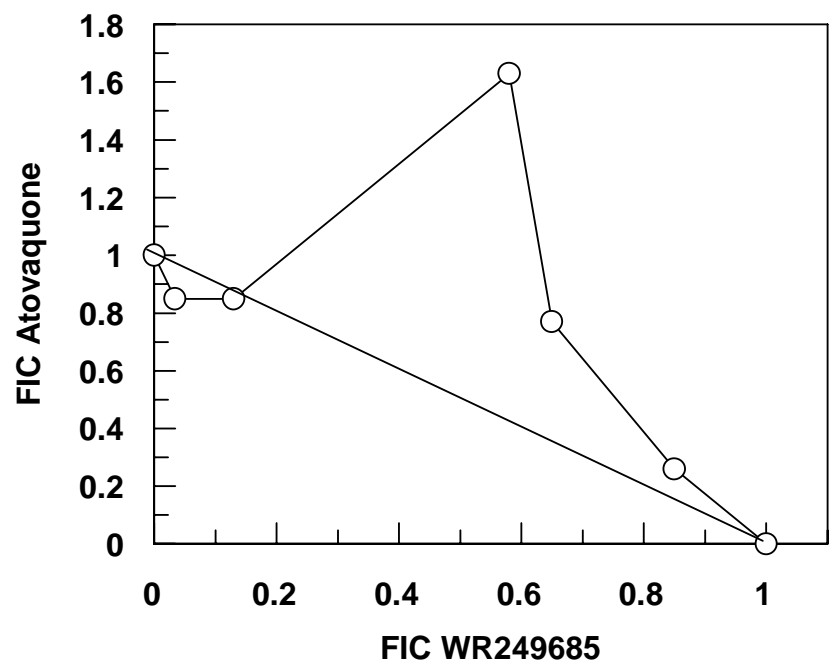
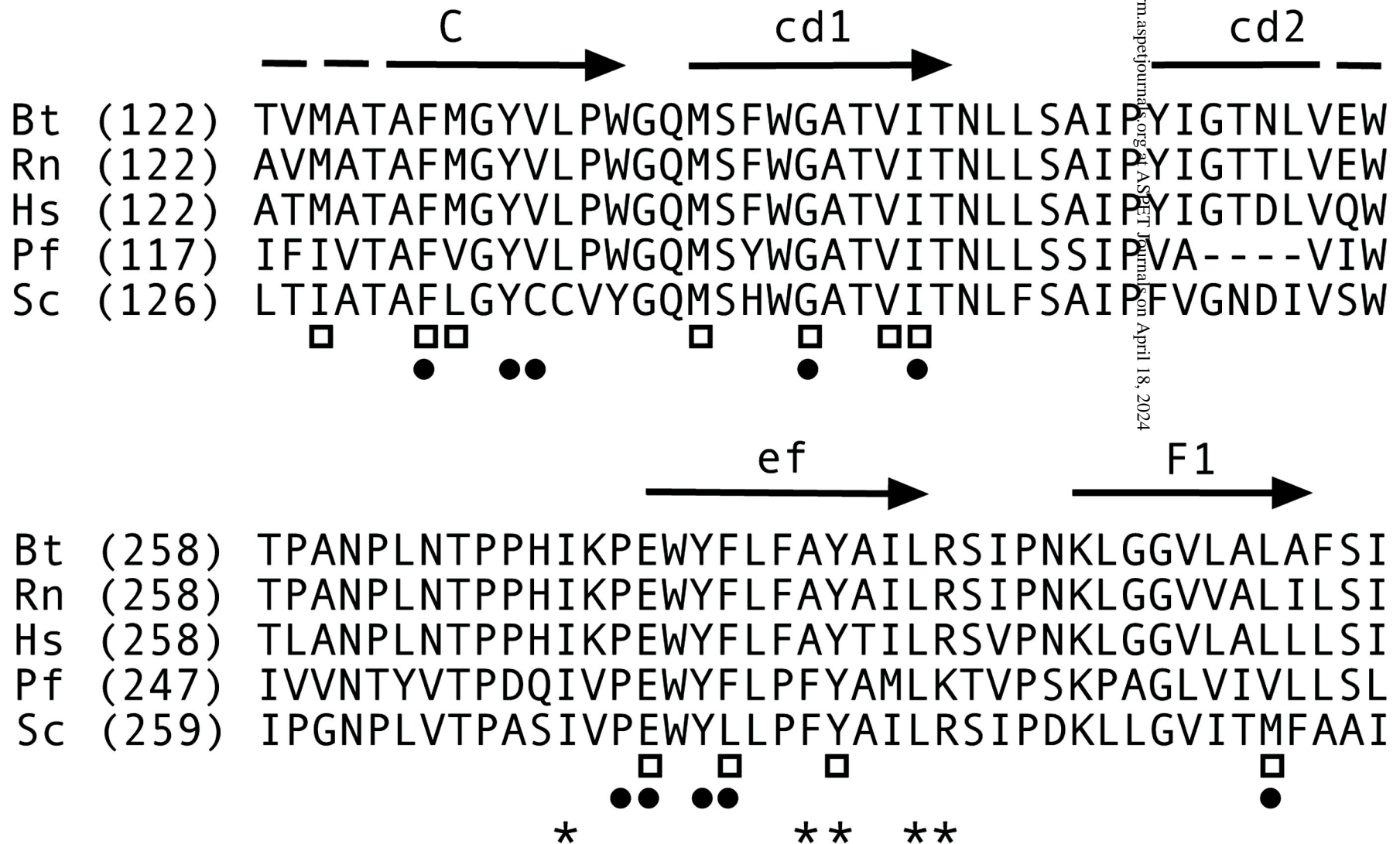


Fig. 4



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Fig. 5A

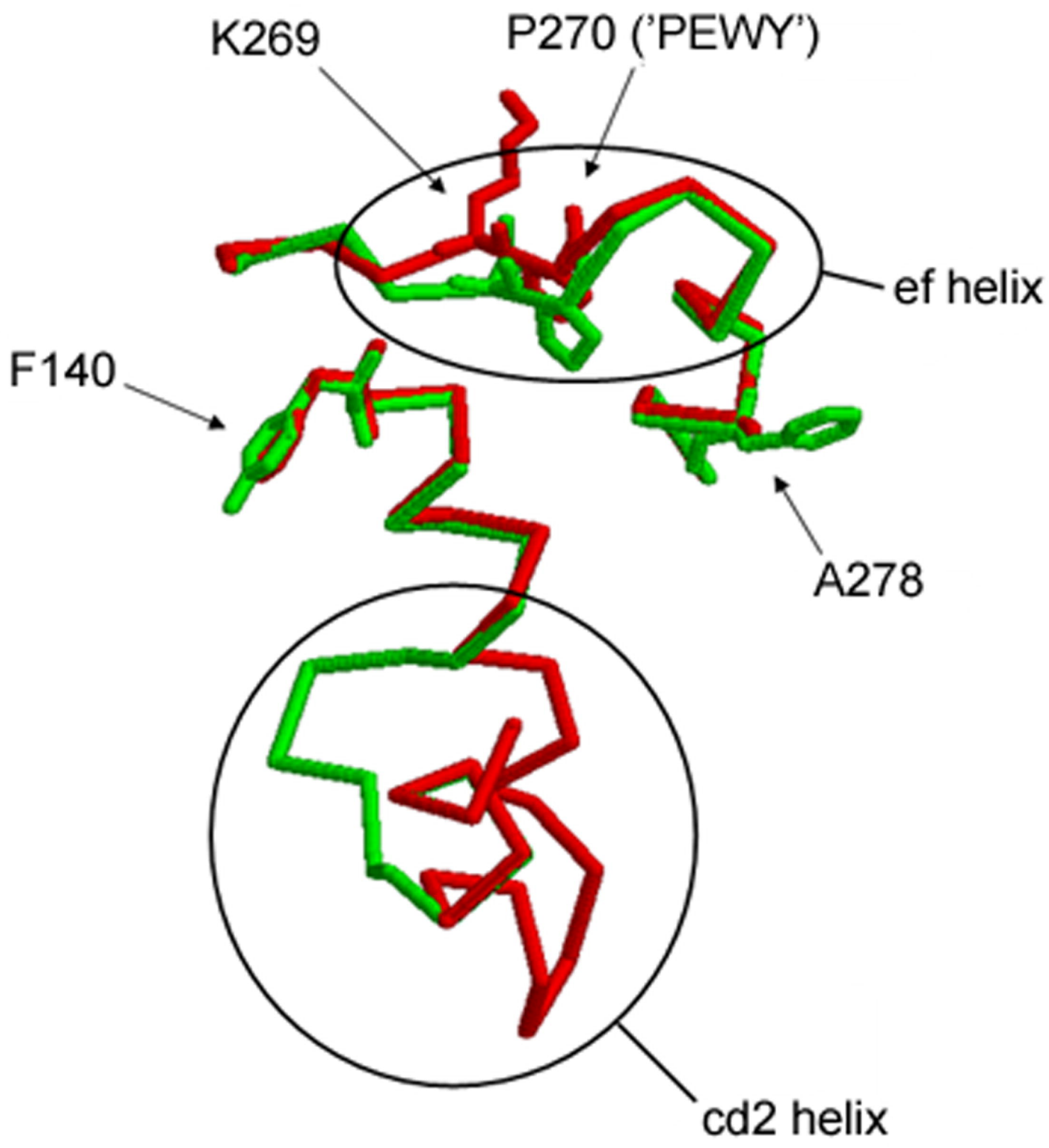


Fig. 5B

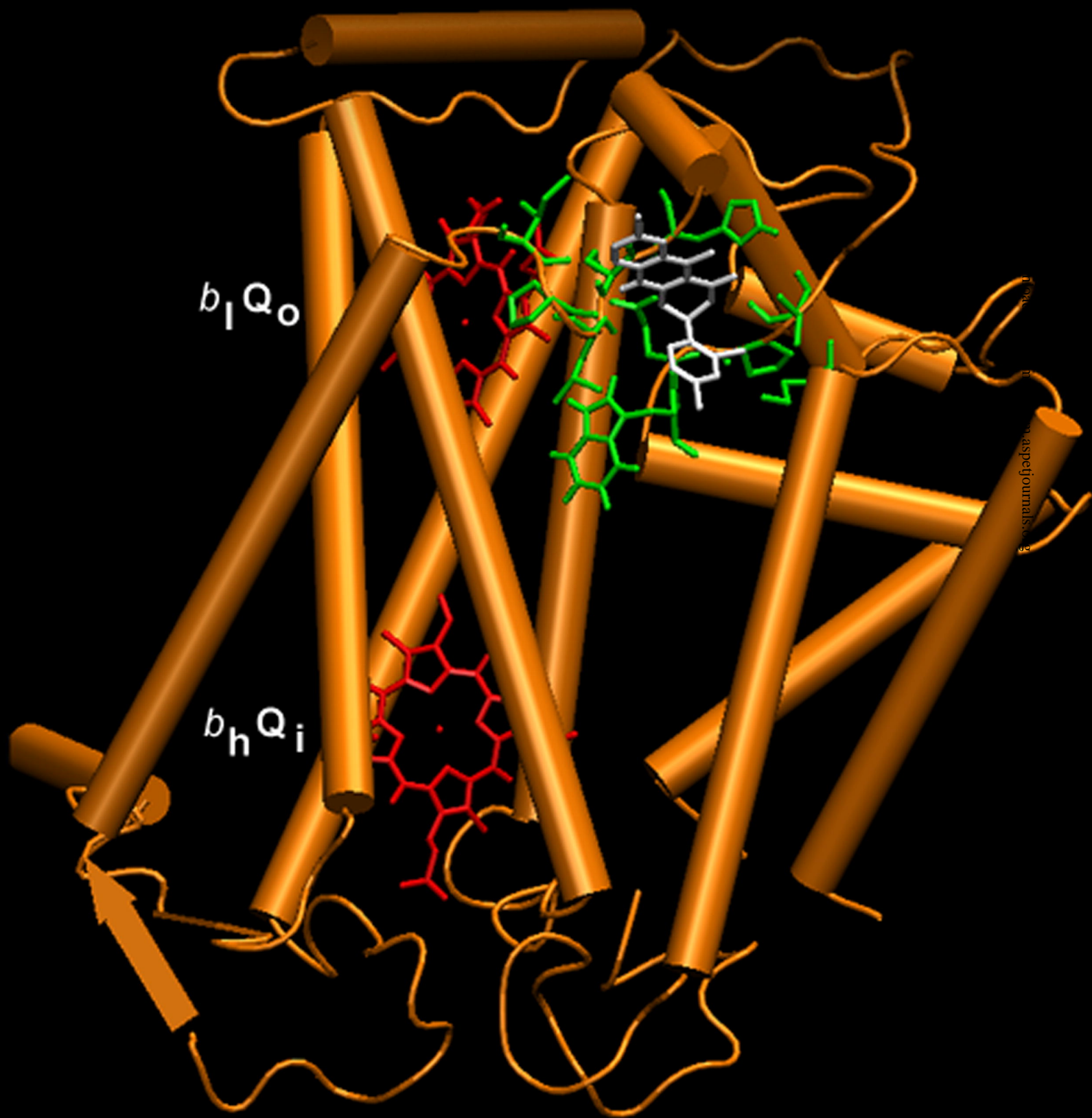


Fig. 6A

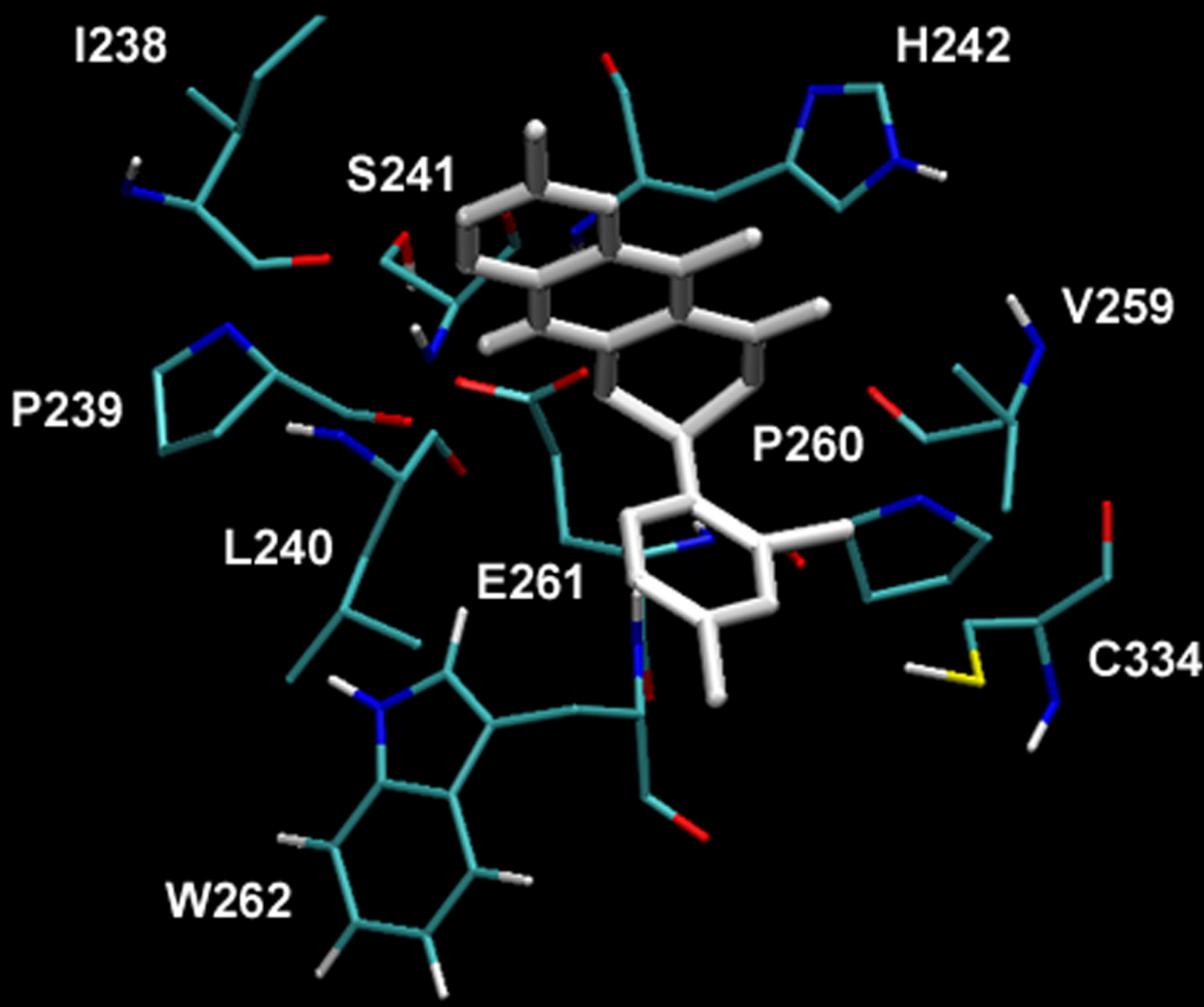


Fig. 6B