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Design of anti-parasitic and anti-fungal hydroxy-naphthoquinones that are less susceptible to drug resistance

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ABSTRACT

Atovaquone is a hydroxy-naphthoquinone that is used to treat parasitic and fungal infections including *Plasmodium falciparum* (malaria), *Pneumocystis jivorecii* (pneumonia) and *Toxoplasma gondii* (toxoplasmosis). It blocks mitochondrial oxidation of ubiquinol in these organisms by binding to the ubiquinol oxidation site of the cytochrome bc_1 complex. Failure of atovaquone treatment has been linked to the appearance of mutations in the mitochondrially encoded gene for cytochrome *b*. In order to determine the optimal parameters required for inhibition of respiration in parasites and pathogenic fungi and overcome drug resistance, we have synthesized and tested the inhibitory activity of novel hydroxy-naphthoquinones against blood stage *P. falciparum* and liver stage *P. berghei* and against cytochrome bc_1 complexes isolated from yeast strains bearing mutations in cytochrome *b* associated with resistance in *Plasmodium*, *Pneumocystis*, and *Toxoplasma*. One of the new inhibitors is highly effective against an atovaquone resistant *Plasmodium* and illustrates the type of modification to the hydroxy-naphthoquinone ring of atovaquone that might mitigate drug resistance.

1. Introduction

Atovaquone (ATV, Fig. 1 A) is a hydroxy-naphthoquinone (NQ) used to prevent and treat infections caused by *Plasmodium falciparum* (malaria) and *Pneumocystis* (pneumonia), a major cause of illness and death in immunocompromised individuals. Ubiquinol oxidation is blocked upon atovaquone binding to the ubiquinol oxidation site of the cytochrome bc_1 complex, which inhibits respiration in these parasites. Although the bc_1 complex is essential for energy transduction in all eukaryotic cells, recent studies indicate that during the erythrocytic stages of growth the essential function of the bc_1 complex in *P. falciparum* is to maintain ubiquinone oxidized and thus available as an electron acceptor for dihydroorotate dehydrogenase [1]. The latter enzyme is essential for pyrimidine biosynthesis in the parasite.

Since the bc_1 complex is essential for ubiquinol reoxidation, and thus indirectly essential for pyrimidine biosynthesis in *Plasmodium*, it is an attractive target for development of novel anti-malarials. This has led to the design of several new bc_1 inhibitors, such as the 4-pyridone derivatives [2,3] including GW844520 [4], and several novel hydroxy-naphthoquinones [5], all of which bind to the ubiquinol oxidation site in the bc_1 complex and are effective at low nM concentrations against the parasite.

Malarone® is a drug combination containing atovaquone and proguanil, an inhibitor of dihydrofolate reductase, which is also essential for pyridimine biosynthesis in the parasite. Spontaneously arising mutations conferring resistance to atovaquone have been linked to the mitochondrially encoded gene for cytochrome *b* [6], and reports of Malarone® treatment failure describe mutations at codon 268 of the cytochrome *b* gene that result in substitution of Tyr with Asn, Ser or Cys in cytochrome *b* in *P. falciparum* [7].

Since atovaquone also inhibits the yeast cytochrome bc_1 complex, we have previously developed *Saccharomyces cerevisiae* as a model to study resistance conferring cytochrome *b* mutations such as Y279S (which corresponds to Y268S in *Plasmodium*), L275F (L248F in *Pneumocystis* numbering) and M139L (which corresponds to M129L in *T. gondii*) [8–11]. Using this model system we were able to show that the mutations at Tyr-268 in *Plasmodium* disrupt non-covalent interactions between atovaquone and the binding pocket in the bc_1 complex [8], and that the most commonly observed mutation in atovaquone resistant *Pneumocystis*, L248F, creates an unfavorable bulge in the binding pocket [9]. These structural changes explain how these mutations impair the efficacy of the drug.

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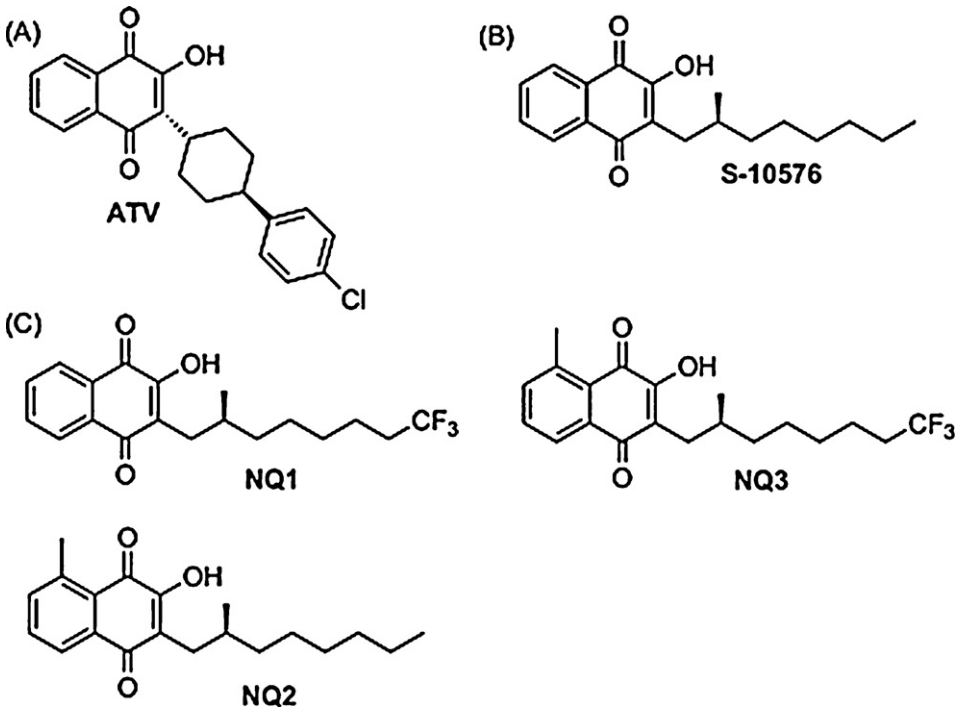

Fig. 1. (A) Atovaquone (B) S-10576 (C) 2-OH-3-(2-methyl-trifluorooctyl)-naphthoquinone (**NQ1**), 2-OH-3-(2-methyl-octyl)-8-methyl-naphthoquinone (**NQ2**) (**NQ3**), 2-OH-3-(2-methyl-trifluorooctyl)-8-methyl-naphthoquinone.

We have recently synthesized the stereochemically active derivative of a potent cytochrome bc_1 complex inhibitor S-10576 (Fig. 1B), featuring a metabolically stable trifluoromethyl function and a methyl substituted aromatic ring (Fig. 1C and Refs 12, 13). The racemic mixture of R- and S-10576 had first been tested by Fieser and coworkers as an anti-malarial [14], who showed that although it was effective against avian malaria, it was ineffective in humans. It was then shown that R/S-10576 is excreted as a water-soluble derivative in which the terminal methyl group is removed from the 2-methyl-octyl side chain by oxidative decarboxylation to yield a 2-methyl-heptyl carboxylic acid side chain [15]. We reasoned that a trifluoromethyl group at the terminus of the side-chain should block this detoxification reaction.

The 8-methyl group was introduced into the naphthoquinone ring in NQ2 and NQ3 on the basis of molecular modeling that indicated it should be possible to create non-covalent interaction between the ligand and the Rieske iron-sulfur protein by this modification. We expected that interaction with this nuclear encoded protein would lessen the frequency of spontaneously arising resistance conferring mutations that otherwise are directed to weakening interactions between the mitochondrial encoded cytochrome *b*, which is more prone to spontaneously arising mutations than a nuclear gene.

Here we report the activity of these hydroxy-naphthoquinones ("NQ's") against the malaria parasite and cytochrome bc_1 complexes isolated from wild-type yeast, and yeast carrying Y279S, L275F and M139L cytochrome *b* mutations in order to determine the parameters required for inhibition of drug resistant parasites and fungi.

2. Experimental procedures

2.1. Materials

Atovaquone was a gift from GlaxoSmithKline. The hydroxy-naphthoquinones S-10576 (13) and the NQ1-3 [16] were synthesized as previously described. The NQ's were dissolved in dimethyl sulfoxide at 2 mM concentration and stored at −20 °C.

2.2. Isolation of yeast mitochondrial membranes

The yeast strains with wild-type background and cytochrome *b* mutations L275F, Y279S and M139L were grown in 2% YPD media as detailed by Kessl et al. [8,11,17]. Mitochondrial membranes were prepared by breaking yeast cells with glass beads in a bead beater as described elsewhere [18] with the following modifications. Before breaking the cells, they were suspended in buffer volume equivalent to two times the wet weight of the cell pellet and 1 mM diisopropylfluorophosphate was added to the buffer. An equal volume of glass beads (Sigma, 425–600 μM) was then added and the cells were broken in a glass bead beater and membranes recovered by centrifugation [18]. After washing the membranes with 50 mM Tris, pH 8.0, the membranes were stored at −20 °C in 50 mM Tris, pH 8.0, containing 50% glycerol. Quantification of the bc_1 complex was performed spectrophotometrically [19], using extinction coefficients of 17.5 mM^{-1} cm^{-1} at 553–539 for cytochrome c_1 [20] and 25.6 mM^{-1} cm^{-1} at 563–579 for the average absorbance of the b_H and b_L hemes in cytochrome *b* [21].

2.3. Ubiquinol-cytochrome c reductase activity measurements

Cytochrome *c* reductase activities of membrane preparations were assayed in 50 mM potassium phosphate, pH 7.0, 250 mM sucrose, 2 mM EDTA, 1 mM NaN_3, 2.5 mM KCN, 0.01% dodecylmaltoside, and 40 μM cytochrome *c* at 23 °C. The mitochondrial membranes were diluted to a cytochrome bc_1 complex concentration of approximately 5 nM in the assay buffer, inhibitor was added to the assay mixture and allowed to stir with the enzyme for 1 min, after which the reaction was started by adding 50 μM 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinol, an analogue of ubiquinol. Reduction of cytochrome *c* was monitored in an Aminco DW-2a spectrophotometer at 550 versus 539 nm in dual wavelength mode. Data were collected and analyzed using an Online