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Parameters determining the relative efficacy of hydroxy-naphthoquinone inhibitors of the cytochrome bc_1 complex

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Abstract

Hydroxy-naphthoquinones are competitive inhibitors of the cytochrome bc_1 complex that bind to the ubiquinol oxidation site between cytochrome *b* and the iron–sulfur protein and presumably mimic a transition state in the ubiquinol oxidation reaction catalyzed by the enzyme. The parameters that affect efficacy of binding of these inhibitors to the bc_1 complex are not well understood. Atovaquone®, a hydroxy-naphthoquinone, has been used therapeutically to treat *Pneumocystis carinii* and *Plasmodium* infections. As the pathogens have developed resistance to this drug, it is important to understand the molecular basis of the drug resistance and to develop new drugs that can circumvent the drug resistance. We previously developed the yeast and bovine bc_1 complexes as surrogates to model the interaction of atovaquone with the bc_1 complexes of the target pathogens and human host. As a first step to identify new cytochrome bc_1 complex inhibitors with therapeutic potential and to better understand the determinants of inhibitor binding, we have screened a library of 2-hydroxy-naphthoquinones with aromatic, cyclic, and non-cyclic alkyl side-chain substitutions at carbon-3 on the hydroxy-quinone ring. We found a group of compounds with alkyl side-chains that effectively inhibit the yeast bc_1 complex. Molecular modeling of these into the crystal structure of the yeast cytochrome bc_1 complex provides structural and quantitative explanations for their binding efficacy to the target enzyme. In addition we also identified a 2-hydroxy-naphthoquinone with a branched side-chain that has potential for development as an anti-fungal and anti-parasitic therapeutic.
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Keywords: Hydroxy-naphthoquinones; Cytochrome bc_1 complex; Malaria; *Plasmodium*; *Pneumocystis*; Atovaquone

1. Introduction

Atovaquone is a substituted hydroxy-naphthoquinone (2-[*trans*-4-(4′-chlorophenyl) cyclohexyl]-3-hydroxy-1,4-naphthoquinone) that binds tightly and competitively to the ubiquinol oxidation site of the cytochrome bc_1 complex [1]. This inhibitor was first used therapeutically as an anti-malaria compound with broad spectrum activity against apicomplexan parasites [2–4] and later was also shown to prevent and clear *Pneumocystis jirovecii* pneumonia [5,6]. However, the pathogens soon developed resistance to the drug, and it was shown that the resistance was due to mutations in the gene for cytochrome b, a subunit that forms part of the ubiquinol oxidation site of the cytochrome bc_1 complex [7]. As the yeast bc_1 complex is also inhibited by atovaquone, we have developed the yeast *Saccharomyces cerevisiae* as a model to explain atovaquone resistance in *P. jirovecii* [8], *Plasmodium falciparum* [9] and *Toxoplasma gondii* [10].

In the current study, we have extended the use of the yeast bc_1 complex model by combining inhibitor titrations and computer modeling calculations to understand the structural parameters that affect efficacy of binding of 2-hydroxy-naphthoquinones to the bc_1 complex. We first screened a randomly chosen library of 2-hydroxy-naphthoquinones with aromatic, cyclic, and non-cyclic alkyl side-chains at position 3 on the hydroxy-quinone ring for inhibition of bc_1 complex activity. We then tested a series of 2-hydroxynaphthoquinones

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with linear alkyl side-chains of varying length in order to obtain a quantitative structure/activity relationship based on side-chain length. We also evaluated the relative efficacy of two stereoisomers with a branched side-chain. The results of these comparisons provide a starting point to synthesize new hydroxy-naphthoquinone inhibitors of the bc_1 complex with potential therapeutic uses.

2. Experimental procedures

2.1. Materials

Dodecylmaltoside was obtained from Roche Applied Science. DEAE-Biogel A was obtained from Bio-Rad. Diisopropylfluorophosphate, decyl ubiquinone and dithionite were purchased from Sigma. Stigmatellin was purchased from Fluka Biochemica. Atovaquone was a gift from GlaxoSmithKline. A collection of 2-hydroxy-1,4-naphthoquinones with aromatic, cyclic, and non-cyclic alkyl side-chains was obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis of the National Cancer Institute. The hydroxy-1,4-naphthoquinones were dissolved in dimethyl sulfoxide at 2 mM concentration and stored at −20 °C.

2.2. Synthesis of naphthoquinones

The synthesis of 3-alkyl-2-hydroxy-1,4-naphthoquinones exploits radical alkylation of commercial 2-hydroxy-1,4-naphthoquinone with alkyl iodides in the presence of tributyltin hydride and 2,2′-azobisisobutyronitrile as radical initiator [11]. $PhSO_2$- protection with $PhSO_2Cl/K_2CO_3$ in DMF gave 66% yield of the *O*-protected product. Chiral (S)- and (R)-2-methyloctyl iodides were prepared in accordance with the known sequence [12–14], which started from methyl-(R)- or (S)-3-hydroxy-2-methylpropionate correspondingly and included copper catalyzed cross coupling of alkyltosylate and Grignard reagent as a key step [15]. The synthesized chiral iodides were then used for radical alkylation.

2.3. Purification of cytochrome bc_1 complexes

Cytochrome bc_1 complexes from yeast and bovine heart were isolated from mitochondrial membranes as described previously [16,17].

2.4. Ubiquinol-cytochrome c reductase activity measurements

Cytochrome c reductase activity was assayed in 50 mM potassium phosphate, pH 7.0, 250 mM sucrose, 0.2 mM EDTA, 1 mM NaN_3, 2.5 mM KCN, 0.01% Tween-20 and 40 μM cytochrome *c* at 23 °C. The cytochrome bc_1 complex was diluted to 2.5 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 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 Instrument Systems Inc. computer interface and software. IC_{50} values for compounds that were inhibitory at concentrations less than 200 nM were determined from titration curves of inhibitor concentration versus activity. Relative amounts of the chiral isomers in the racemic mixture of compound #10576 were calculated from the IC_{50} values of the two isomers and that of the mixture using the equation $X/IC_{50(S)}+Y/IC_{50(R)}=1/IC_{50(M)}$ where $X+Y=1.0$.

2.5. Molecular modeling

Molecular modeling was carried out on Silicon Graphics O2 and Octane workstations using the commercially available Insight II® software package (Acclerys Inc., San Diego). The starting structure was the energy-minimized atovaquone-liganded yeast cytochrome bc_1 complex [1]. Briefly, a minimized conformation of atovaquone was docked into a stigmatellin liganded crystal structure [18] that was modified to include the rotation of Glu-272 and a water mediated hydrogen bond observed in the nHDBT liganded crystal structure [19]. For the structures shown here, the initial docking was achieved by overlay of the hydroxynaphthoquinone ring with the one from the previously calculated atovaquone structure. The initial position of the linear aliphatic side-chain, prior to the molecular dynamics run, followed the one observed in the nHDBT crystal structure.

For the molecular dynamics calculations, a set of restraints was set up between the hydroxy-naphthoquinones, His-181 of the Rieske protein, and Glu-272 of cytochrome *b* in order to maintain the ligand in position during the dynamic stages. The naphthoquinones and cytochrome *b* residues within 4.0 Å were allowed to be flexible. A surrounding 9.5 Å shell of residues in both cytochrome *b* and the iron–sulfur protein was fixed, and the most distant residues were excluded from the calculation in order to obtain a manageable simulation speed. A 9.5 Å atom-based cut-off for nonbonding interactions was used during the calculations, with the dielectric constant set at 2.0. Eight simulated annealing runs were performed, each from 800 to 298 K, with five temperature steps and a simulation time of 5000 fs/step. The Nose temperature control method was used with a 0.5 fs/iteration time step. A custom macro was written to select the lowest energy structure from each dynamics run for continued modeling. Between each dynamics run, a minimization of 250 iterations was performed. After the final round of molecular dynamics, the lowest energy structure was minimized to a final convergence criterion of 0.001, using Cauchy's steepest descent method as implemented in the Discover 3® module within the Insight II® software, followed by conjugate gradient and Newton methods in succession. Of the eight minimized results obtained, the three lowest energy structures were chosen for binding energy calculation.

The binding energy calculation was adapted from a previous method [8] and uses a common subset that included the naphthoquinone and cytochrome *b* residues within 4.0 Å of the inhibitor. The reported value for each naphthoquinone is an average of the three calculated lowest energy structures and contains non-bonding interactions (van der Waals and electrostatic) as well as internal conformational energies of the ligand and adjacent pocket residues.

3. Results

3.1. Inhibition of bovine and yeast bc_1 complexes by linear alkyl Naphthoquinones

The molecular target of the hydroxy-naphthoquinone inhibitors is known to be the ubiquinol oxidation pocket at the center P site of the cytochrome bc_1 complex [1]. We thus tested a library of approximately 60 2-hydroxy-naphthoquinones for inhibition of ubiquinol-cytochrome *c* reductase activity of both yeast and bovine cytochrome bc_1 complexes. The compounds tested included derivatives with aromatic, cyclic non-aromatic, branched and linear side-chains at the carbon-3 position of the hydroxy-quinone ring. The results of this initial survey are summarized in Supplemental Data Table 1, which also includes the structures of the tested compounds. Most of these compounds were found to show no or very poor bc_1 complex inhibition and were not studied further.

A small group of alkyl hydroxy-naphthoquinones displayed significant inhibition of enzymatic activity. We thus synthesized the remaining compounds of the series with linear side-chains of varying length for a more systematic study of the structure/activity relationship in this group of inhibitors. All members of the linear series inhibited both yeast and bovine cytochrome bc_1 complex activities. As can be seen from the data in Fig. 1, the

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