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Phthalimide Derivatives with Bioactivity against *Plasmodium falciparum*: Synthesis, Evaluation, and Computational Studies Involving bc_1 Cytochrome Inhibition

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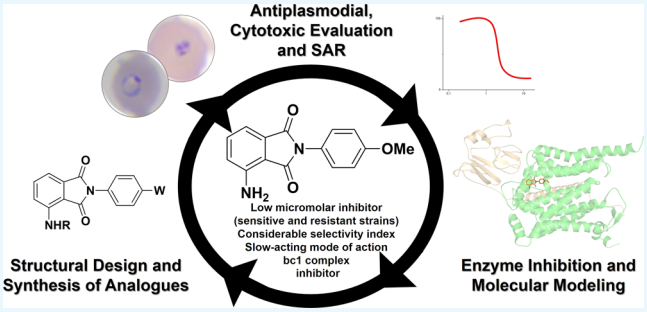
Supporting Information

ABSTRACT: We describe herein the design and synthesis of *N*-phenyl phthalimide derivatives with inhibitory activities against *Plasmodium falciparum* (sensitive and resistant strains) in the low micromolar range and noticeable selectivity indices against human cells. The best inhibitor, 4-amino-2-(4-methoxyphenyl)isoindoline-1,3-dione (**10**), showed a slow-acting mechanism similar to that of atovaquone. Enzymatic assay indicated that **10** inhibited *P. falciparum* cytochrome bc_1 complex. Molecular docking studies suggested the binding mode of the best hit to Qo site of the cytochrome bc_1 complex. Our findings suggest that **10** is a promising candidate for hit-to-lead development.



INTRODUCTION

Malaria is a severe parasitic disease endemic in 91 countries. According to the WHO, 216 million cases and approximately 445.000 deaths globally were reported in 2016.[1] The disease can be caused by five species of Plasmodium spp (*Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae,* and *Plasmodium knowlesi*). *P. falciparum* is the major pathogen responsible for deaths, and it is associated with severe malaria.[2] The malaria parasite has developed a resistance to many of the current drugs, including emerging *P. falciparum* resistance to artemisinin derivatives (**1**), which is a component of artemisinin-based combination therapies that comprise the current first-line therapies.[3] In this context, new safe and affordable antimalarial drugs are desperately needed. Structure-based approaches have been used to develop new antimalarial candidates with well-defined mechanisms of action.[4−6] The cytochrome bc_1 complex contains a coenzyme Q, a cytochrome *c* reductase, and the ligand ubiquinone. The enzymatic complex catalyzes electron transfer in the mitochondria of the parasite, which maintains a stable membrane potential for this organelle.[7,8] Atovaquone (**2**) (Chart 1), a ubiquinone analogue, was approved as a first line drug for the treatment of malaria in 1992.[9] Atovaquone has a well-defined mechanism of action; it binds to the oxidation site of the cytochrome bc_1 enzyme complex.[2,8,10] As a competitive inhibitor of ubiquinone, the inhibitor blocks cellular respiration because of the reduction of electron transfer in the mitochondria of Plasmodium spp, which consequently promotes a collapse in the maintenance of the mitochondrial membrane potential.[10] The ubiquinone binding mode indicates that the molecule interacts with a P center of the bc_1 enzymatic complex, inducing a conformational change in the Rieske protein at Fe−S bond, which allows the electron transfer. The atovaquone mode of action consists of the inhibition of the bc_1 complex, resulting in conformational changes that can affect the electronic transfer to the cytochrome *c*.[9,11]

Recent studies have shown that the inhibition of the bc_1 complex also affects the electron acceptor enzyme known as dihydroorotate dehydrogenase (DHODH). This enzyme plays

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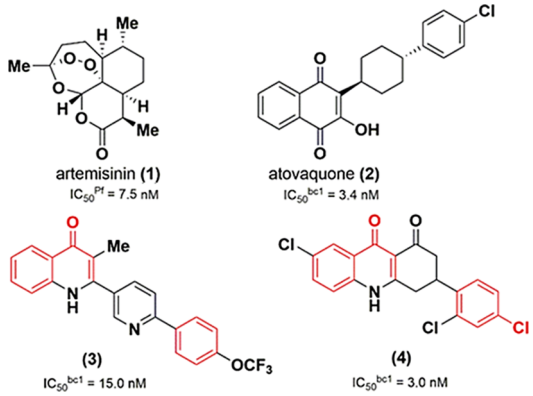
Chart 1. Approved Drugs for the Treatment of Malaria: Artemisinin (1) and Atovaquone (2); 3 (SL-2-25) and 4 (WR249685): Potent Compounds with in Vitro Inhibitory Activity against Whole Parasite (1) or the bc_1 Complex (2–4), and Their Similarities to 2 Are Highlighted in Red

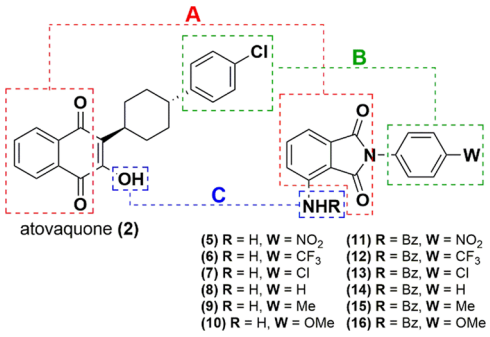


an important role in the biosynthesis of pyridines; therefore, inhibiting this enzymatic complex indirectly impacts the biosynthesis of pyridines in the parasite as well as the transport of electrons in the mitochondria.[9,12]

Genetic mutations causing atovaquone resistance have been described since the early 1990s.[13,14] The replacement of Tyr268 with serine (Y268S) or asparagine (Y268N) disrupts the chemical interactions between atovaquone and the active site residues of the bc_1 complex. Therefore, the investigation of new inhibitors is necessary to reduce the potential for drug resistance. Compounds **3** (SL-2-25) and **4** (WR249685) are examples of atovaquone analogues that are potent bc_1 complex inhibitors (Chart 1).[15,16]

Here, we designed *N*-phenyl-substituted phthalimides (**5**–**16**) (Scheme 1) as surrogates for the atovaquone core. The

Scheme 1. Structural Design of the *N*-Phenyl Phthalimide Fragments from Atovaquone

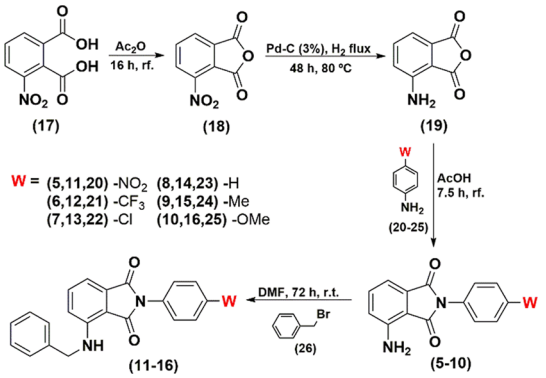


approach consisted of the maintenance of the two aromatic subunits in addition to the 1,4-naphthoquinone moiety (A) including a para-substituted phenyl ring (B) with different electronic and steric properties. The 3-hydroxy group on the 1,4-naphthoquinone moiety was replaced with a monosubstituted amine (C) to maintain the hydrogen-bonding abilities. Atovaquone has shown better oral bioavailability when the 3-hydroxyl substituent was replaced with a hydrophobic group (OCH_3),[11] and a benzyl group (C) was introduced to investigate its impact on the biological properties. It is worth mentioning that the designed compounds (**5**–**16**) follow the rule of three for fragment-based drug discovery, that is, a molecular weight lower than 300 Da, up to three hydrogen bond acceptors and a calculated log $P < 3$.[17]

RESULTS AND DISCUSSION

In the synthesis of the *N*-phenyl-substituted phthalimide derivatives (**5**–**16**) (Scheme 2), 3-nitrophalic acid (**17**) was

Scheme 2. Synthetic Route Used to Obtain the *N*-Phenylphthalimides (5–16)



refluxed in acetic anhydride for 16 h.[18] Then, the nitro group of anhydride **18** underwent catalytic hydrogenation with Pd/C (3%) resulting in **19**.[19] In a divergent synthetic step, 3-aminophalic anhydride (**19**) was refluxed for 7.5 h in glacial acetic acid with the appropriate para-substituted aniline (**20**–**25**) to afford fragments **5**–**10**.[20] Finally, these aminophthalimides (**5**–**10**) were dissolved in *N*,*N*-dimethylformamide with benzyl bromide (**26**) to form the *N*-benzylphthalimides (**11**–**16**).[21] See the Supporting Information for more details regarding the experimental procedures and the characterization data of the products and intermediates.

Compounds **5**–**16** were evaluated in vitro against *P. falciparum* (3D7 strain—chloroquine sensitive) by the SYBR green assay.[22] The compounds were tested at single concentration (10 μM), and IC_{50} values were determined for compounds that showed inhibitions >60%. Compounds **5**–**9** and **11**–**15** were inactive as they presented IC_{50}^{Pf} values higher than 10 μM (Table 1).

Conversely, compounds **10** and **16** inhibited in vitro *P. falciparum* growth at low micromolar concentrations (IC_{50}^{Pf} values of 4.2 and 6.8 μM, respectively). These data suggest that a para-methoxy substituent is beneficial for the inhibitory activity in this series. Moreover, the bulky benzyl substituent at site C was tolerated, suggesting that structural variation at this position can be further explored. Next, the cytotoxic effects of the active compounds were evaluated against a human hepatoma cell line (HepG2) by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide viability assay.[23] Compounds **10** and **16** showed low cytotoxicities (IC_{50}^{HepG2} > 250 μM) and considerable selectivity indices (SI values of >36 and >59, respectively) (Table 1). It is worth mentioning that both compounds were soluble at the highest concentration tested (250 μM).

To better characterize the antiplasmodial activities and confirm the low cytotoxicities of the series, the most potent phthalimide derivative (**10**) was selected. The morphology of HepG2 cells was evaluated after 24 h of treatment with 250 μM of **10**. At this concentration, we verified that compound **10**

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Table 1. Inhibitory and Cytotoxic Activities of the Phthalimide Derivatives against *P. falciparum* (3D7 Strain) and HepG2 Cells[a]

Cpd	% of inhibition @ 10 μM	IC_{50}^{Pf} (CI95%) (μM)	IC_{50}^{HepG2} (μM)	SI*
5	15 ± 2	>10	nd	nd
6	3 ± 2	>10	nd	nd
7	5 ± 3	>10	nd	nd
8	6 ± 3	>10	nd	nd
9	4 ± 2	>10	nd	nd
10	75 ± 4	4.2 (3.2−5.2)	>250	>59
11	6 ± 2	>10	nd	nd
12	5 ± 2	>10	nd	nd
13	7 ± 3	>10	nd	nd
14	23 ± 3	>10	nd	nd
15	8 ± 3	>10	nd	nd
16	65 ± 5	6.8 (5.6−8.0)	>250	>36
artesunate		0.006 (0.005−0.008)	305 (281−329)	50.833

[a]The presented IC_{50} values are the average of two independent experiments. *SI = $IC_{50}^{HepG2}/IC_{50}^{Pf}$; CI95% = 95% confidence interval; nd = not determined.

was not toxic to the cells as they showed a microscopic morphology similar to that of untreated cells (Figure 1A).

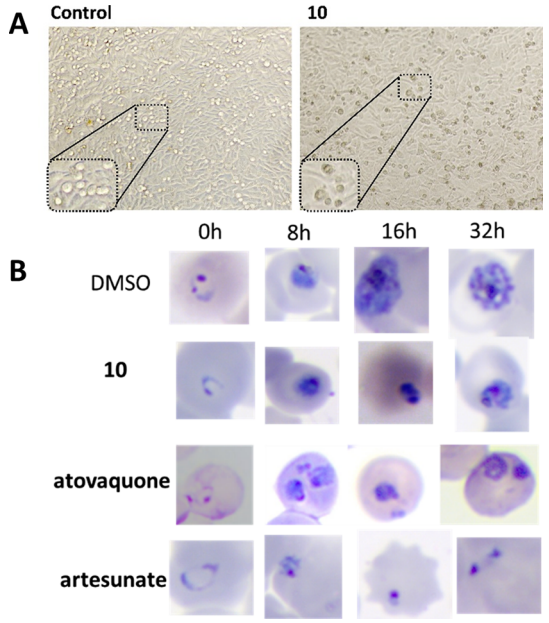

Figure 1. (A) Morphology of HepG2 cells before and after incubation with 10 at 250 μM. Insets: Expansion showing representative cell morphology; (B) microscopy of synchronized parasites continuously treated with **10** at 40 μM and the controls [dimethyl sulfoxide (DMSO), **10**, atovaquone, and artesunate]. Representative images of two independent experiments.

Next, the inhibitory activity of **10** was evaluated against the *P. falciparum* K1 strain, a strain that is multidrug-resistant to the antimalarial drugs chloroquine, pyrimethamine, and sulfadoxine. Compound **10** was active in the low micromolar range against the resistant parasite with an IC_{50} value of 4.3 μM, which is approximately the same inhibitory activity observed against the sensitive 3D7 strain (Figure 2). To elucidate the stage-specific inhibitory activity of **10**, we incubated the compound with highly synchronized parasites at a concentration 10-fold greater than its IC_{50} value and observed the morphological changes over time. As seen in Figure 1B, compound **10** showed activity in the later ring

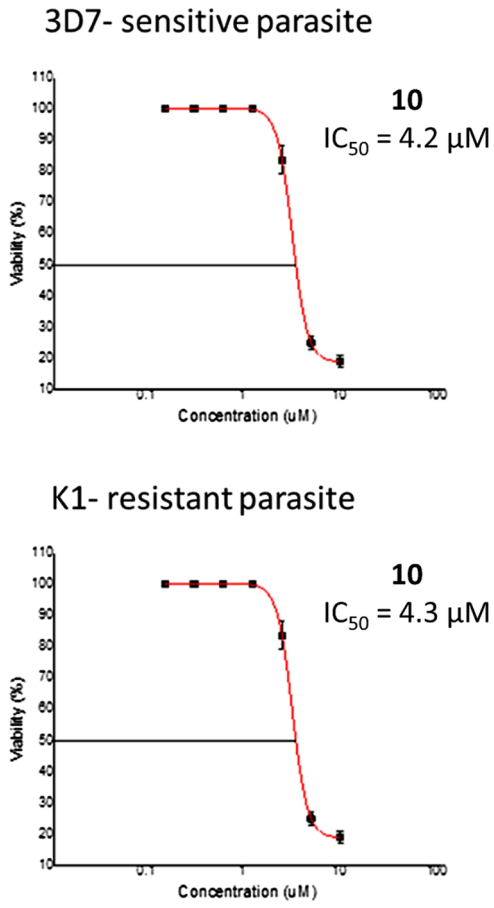

Figure 2. Representative concentration−response curves of **10** against sensitive (3D7 strain) and resistant parasites (K1 strain). The reported IC_{50} values are the mean values of two independent experiments.

stages (late rings or early trophozoites) and it induced alterations in *P. falciparum* morphology between 16 and 32 h after incubation. These data suggest a stage-specific effect that may be related to a slow-acting mechanism in which the latter forms of *P. falciparum* in the intraerythrocytic cycle are susceptible to the effects of the compound. These results are in

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good agreement with the stage-specific inhibitory activity of atovaquone, which is a slow-acting inhibitor of *P. falciparum*.[24]

Aiming to elucidate the effects of compound **10** on *P. falciparum* mitochondrial respiration, an enzymatic assay was conducted to measure bc1 complex decylubiquinol-cytochrome *c* oxidoreductase activity.[25] Mitochondrial fractions were extracted from *P. falciparum* 3D7 parasites, and the assay was performed using a compound **10** concentration of 70 μM, in parallel with negative (DMSO) and positive (atovaquone at 6 μM) controls. Our results showed that **10** inhibited cytochrome *bc*1 enzymatic activity (74% at 70 μM) and demonstrated that cytochrome *bc*1 complex is a molecular target of **10** antiplasmodial activity (Figure 3).

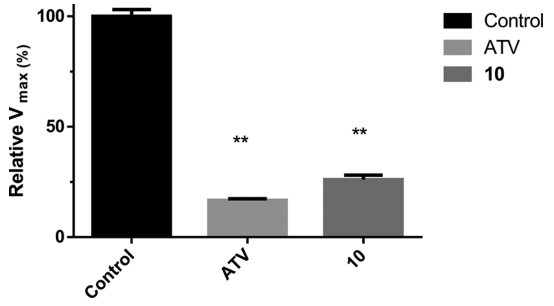

Figure 3. Inhibition of bc1 complex activity after incubation with atovaquone (ATV) at 6 μM (84 ± 1% of inhibition) or compound **10** at 70 μM (74 ± 2% of inhibition). DMSO was used as a negative control (1 ± 3% of inhibition). $^{**}p < 0.005$ by Mann−Whitney test.

The mode of atovaquone binding to the cytochrome bc_1 complex indicated that the carbonyl group alpha to the hydroxyl group is important for ligand binding and recognition. In addition, the hydroxyl group is involved in a salt bridge with the nitrogen atom of the imidazole moiety of histidine in the Rieske protein.[10,11] Finally, hydrophobic interactions and van der Waals forces are important for stabilizing atovaquone binding. For example, the cyclohexyl subunit and the 4-chlorophenyl substituent interact with the side chains of the V146, I269, P271, M139, L275, M295, and F296 residues from the *Saccharomyces cerevisiae* cytochrome bc_1 complex.[26] Accordingly, on the basis of the structural similarities and in vitro cellular findings, which indicated that phthalimide derivative **10** shares a slow-acting inhibitory mechanism with atovaquone, we modeled the binding mode of **10** to the cytochrome bc_1 complex.

The crystal structure of the *S. cerevisiae* cytochrome bc_1 complex co-crystallized with stigmatellin was solved at 1.9 Å resolution (PDB ID: 3CX5)[27] and has been used as a molecular target for modeling studies. The observed binding mode of atovaquone co-crystalized with a cytochrome bc_1 complex (PDB ID: 4PD4, resolution: 3.04 Å)[26] was used as a reference. Superposition of the two crystal structures showed no significant differences in the orientation of the amino acid residues within the protein binding sites, suggesting that the higher resolution structure is adequate for docking studies. The cytochrome bc_1 complex from *S. cerevisiae* was prepared for rigid docking using GOLD software (v5.4).[28] The hydrogen atoms were added to the structure, and the water molecules and ligands were removed. The 3D structure of the hydroxyl group of atovaquone was built in the ionized state because previous studies showed that this microspecies is responsible for the activity.[26]

To evaluate the accuracy of the method, including the ChemPLP scoring function,[29] we redocked atovaquone into the cytochrome bc_1 complex binding site. The best-ranked binding mode was compared to the binding mode of atovaquone in the crystal structure, and both the orientation and interactions with key residues were reproduced (RMSD = 0.4 Å; PLPscore = 63.3), which confirms the accuracy of this strategy for the system under investigation (see the Supporting Information for details). On the basis of that, we used the validated protocol to predict the binding mode of **10** within the cytochrome bc_1 complex binding site. According to the model, the phthalimidic moiety of **10** establishes hydrogen-bonding interactions with the side chains of E272 and H181. Additionally, this moiety is in close contact with the side chains of I147, V146, and P271 and with the backbone atoms of W142. The methoxyphenyl substituent forms hydrophobic interactions with the side-chain atoms of M295 and L275. The ChemPLP score for this pose is 63.6, which is close to the calculated score for the atovaquone binding mode (Figure 4).

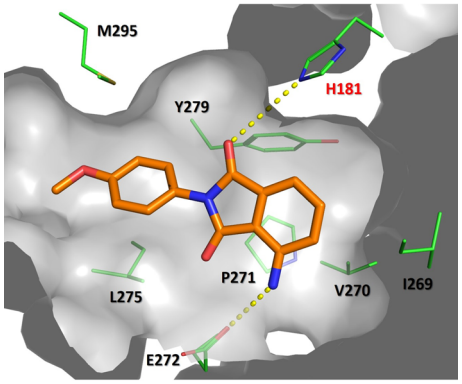

Figure 4. Docking pose of **10** (orange stick) in the binding site of the *S. cerevisiae* cytochrome bc_1 complex (PDB ID 3CX5). Main interacting residues are indicated as green sticks. Hydrogen bonds are represented as yellow dotted lines. Residues I269, V270, P271, E272, L275, Y279, and M295 (black label) are located at the cytochrome *b* subunit, whereas H181 (red label) is located at the Rieske protein subunit. Residues W142, V146, I147, and all hydrogens atoms were omitted for clarity. Figure generated using PyMol (v 0.99).[30]

As highlighted in the sequence alignment (Figure S52),[31,32] all of the mentioned residues interacting with the phthalimidic moiety are conserved in the *P. falciparum* cytochrome bc_1 complex (Uniprot Q02768/Q8IL75), apart from M295 and L275. Residues M295 and L275 from bc_1 of *S. cerevisiae* are replaced with V284 and F264, respectively. In *P. falciparum*, both substitutions can establish hydrophobic interactions with the methoxyphenyl substituent of **10**. Moreover, the alignment of *S. cerevisiae*, *P. falciparum*, and *Mycobacterium tuberculosis* bc_1 sequences shows that the binding site of **10** is conserved within these species. The residues H181, I269, and P271 from bc_1 complex of *S. cerevisiae* are conserved in *P. falciparum* and *M. tuberculosis*, whereas V146 and I147, conserved in *P. falciparum*, are conservative replaced by alanine and leucine, respectively, in *M. tuberculosis*. Most of nonconserved residues within the three species stabilizes compound **10** by hydrophobic and van der Waals interactions. Because a potent antimycobacterial activity is observed for phthalimide-containing compounds whose target is the bc_1 complex of *M.*

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tuberculosis,[33] the binding site identity is an indicative that the bc_1 complex might be the molecular target of compound **10**.

CONCLUSIONS

Phthalimide-based derivatives are attractive molecules for drug discovery and development. Because of the potential biological activity and pharmaceutical use, the privileged phthalimide scaffold was designed to be used in many therapeutic treatments, including antituberculosis as cytochrome bc_1 inhibitor,[33] anti-inflammatory compound developed as a phosphodiesterase-4 inhibitor[34] and for antimalarial as plasmepsin II & IV inhibitor.[35] In this work, the best phthalimide derivative, compound **10**, exhibited antimalarial activity against sensitive and resistant *P. falciparum* strains in the low micromolar range (IC_{50} < 10 μM), no cytotoxicity against HepG2 cells, a considerable selectivity index (SI > 30), and a slow-acting mechanism, which is consistent with the inhibitory stage of action of atovaquone. The modeled binding mode of **10** suggested the molecular determinants that might be related to the inhibitory activity of this series. Therefore, our findings indicate that 4-amino-2-(4-methoxyphenyl)-isoindoline-1,3-dione (**10**) is a new hit for the development of lead compounds with superior properties.

COMPUTATIONAL METHODS

Initially, all molecular structures of the *p*-phenyl-functionalized phthalimidic compounds were generated with Discovery Studio Visualizer free software (DSV/version: 17.2.0)[36] and were optimized using the semiempirical method PM6 in MOPAC free software (version 2016)[37] through the Mercury CSD software interface (v3.9), which is included in the CCDC GOLD Suite computational package. The commands used during optimization were: MMOK, XYZ, CHARGE = −1 [for docking of atovaquone (**2**)] and GNORM = 1. The format of the optimized compound file was *.mol2. Atovaquone, the reference ligand for this work, was modeled in the ionized state.[26] In parallel, the 3D file (*.pdb) of the *S. cerevisiae* cytochrome bc_1 crystal structure in complex with stigmatellin was obtained from Protein Data Bank (PDB ID: 3CX5) at 1.9 Å resolution.[27] The structure was chosen because atovaquone is a high affinity inhibitor of *S. cerevisiae* complex III (K_i = 5 nM), a nonpathogenic surrogate model for studying biological activities of compounds that target the parasite through this mechanism. Although the crystal structure contains a ligand other than atovaquone, we chose it because the cytochrome bc_1 crystal structure in complex with atovaquone (PDB ID: 4PD4)[26] lacks the Rieske protein and has lower resolution in comparison with the 3CX5 structure. Superposition of 3CX5 and 4PD4 indicates no significant differences in the position of the atoms in amino acid residues of the binding site, thereby suggesting that the structure is suitable for the docking calculations using atovaquone's binding mode in 4PD4 as a reference for evaluation of the model. The 3CX5.pdb file was then prepared for docking using GOLD software (v5.4),[28] where the hydrogen atoms were added to the structure. All water molecules and ligands were removed. The rigid docking was performed in GOLD using coordinates x = 13.0858, y = 5.3280, and z = 17.4635 as the center of a 10 Å search radius. GOLD was set to consider possible ligand internal hydrogen bonds in the calculations and score 50 binding modes using the ChemPLP scoring function. The selection of this scoring function was based on a previous article by Li et al. that indicates best accuracy for ChemPLP in comparison with several other score functions.[29] The best GOLD docking results for each ligand were analyzed using Discovery Studio Visualizer free software to check the ligand−cytochrome bc_1 complex interaction types. The figures were generated in Pymol (v0.99).[30]

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b01062.

Syntheses of the phthalimides derivatives **5**−**16**, analytical data, spectra of all synthesized compounds, description of the biological methods for evaluation of antimalarial activity, and computational modeling methodology of the compounds **10** and **16** in bc_1 cytochrome complex (PDF)

Accession Codes

Alphanumeric codes such as Y268S indicate mutations between amino acid residues, and codes such as H181 indicate the amino acid residue and the position in the protein.

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Author Contributions

‖C.Y.O.-J., G.C.M., and A.C.C.A. contributed equally to the work. C.Y.O.-J. and G.C.M. synthesized, isolated, purified, and characterized the compounds. A.C.C.A., G.E.S., and J.O.d.S., performed the in vitro studies. V.S.B., N.M.N.-J., and R.V.B. performed the molecular modeling studies. G.O., N.M.N.-J., R.V.C.G., and V.S.B. conceived the study, analyzed the data, contributed ideas, and wrote the paper.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

HepG2, liver hepatocellular carcinoma; WHO, World Health Organization; log P, log (partition coefficient); Pd/C, palladium on carbon; DMF, *N*,*N*-dimethylformamide; SYBR, *N*′,*N*′-dimethyl-*N*-[4-[(*E*)-(3-methyl-1,3-benzothiazol-2-yl dene)methyl]-1-phenylquinolin-1-ium-2-yl]-*N*-propylpropane-1,3-diamine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-

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tetrazolium bromide; IC_{50}, half maximal inhibitory concentration; LD_{50}, median lethal dose; SI, selectivity index; PM6, parametric method 6; RMSD, root-mean-square deviation

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