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RESEARCH

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Biological evaluation of hydroxynaphthoquinones as anti-malarials

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Abstract

Background: The hydroxynaphthoquinones have been extensively investigated over the past 50 years for their anti-malarial activity. One member of this class, atovaquone, is combined with proguanil in Malarone®, an important drug for the treatment and prevention of malaria.

Methods: Anti-malarial activity was assessed *in vitro* for a series of 3-alkyl-2-hydroxy-1,4-naphthoquinones (N1-N5) evaluating the parasitaemia after 48 hours of incubation. Potential cytotoxicity in HEK293T cells was assessed using the MTT assay. Changes in mitochondrial membrane potential of *Plasmodium* were measured using the fluorescent dye Mitrotracker Red CMXRos.

Results: Four compounds demonstrated IC₅₀s in the mid-micromolar range, and the most active compound, N3, had an IC₅₀ of 443 nM. N3 disrupted mitochondrial membrane potential, and after 1 hour presented an IC_{50Δψ_{mit}} of 16 μM. In an *in vitro* cytotoxicity assay using HEK 293T cells N3 demonstrated no cytotoxicity at concentrations up to 16 μM.

Conclusions: N3 was a potent inhibitor of mitochondrial electron transport, had nanomolar activity against cultured *Plasmodium falciparum* and showed minimal cytotoxicity. N3 may serve as a starting point for the design of new hydroxynaphthoquinone anti-malarials.

Keywords: *Plasmodium falciparum*, Hydroxynaphthoquinone, 2-hydroxy-1,4-naphthoquinone, Mitochondria, Malaria, *Plasmodium berghei*

Background

Despite the worldwide effort to understand molecular and cellular features of *Plasmodium falciparum*, the main aetiological agent of human malaria, the disease is still devastating. Parasite resistance to older anti-malarials raises the need for the development of new drugs [1,2]. The anti-malarials currently used stem from six drug classes: aminoquinolines, arylaminoalcohols, artemisinins, antifolates, antibiotics and inhibitors of the respiratory chain [3,4]. The last class is the subject of this report.

The hydroxynaphthoquinones have been extensively investigated over the past 50 years for their anti-malarial activity [5]. Hydroxynaphthoquinone

discovered that possessed anti-malarial activity [6]. This discovery, which emerged at a time of great interest in the study of hydroxynaphthoquinone derivatives as potential new anti-malarials, resulted in the synthesis of a large family of different hydroxynaphthoquinone analogs [7]. Work on the anti-malarial properties of hydroxynaphthoquinones was revived when chloroquine resistance emerged, and it was discovered that atovaquone effectively inhibits plasmodial electron transport at the ubiquinone (coenzyme Q, 2) site [8].

Atovaquone is a hydroxynaphthoquinone that is used in combination with proguanil (Malarone®) for prophylaxis and therapy of uncomplicated malaria [9]. Atovaquone has excellent anti-malarial activity but exhibits poor pharmaceutical properties, such as low bioavailability and high plasma protein binding [10]. To improve drug bioavailability, several atovaquone analogs were prepared and changes were made to the naphthoquinone moiety, especially the alkyl side chain, because it is known that modifying this

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chain can alter drug activity [7] and counteract drug resistance [11-13]. Recently, it was demonstrated that 2-methyl-heptyl or 2-methyl-heptyl-trifluoromethyl 2-hydroxy-1,4-naphthoquinones were highly effective against atovaquone-resistant *P. falciparum* [14].

The aim of this work was to test the activity of a new series of hydroxynaphthoquinones [15] against *P. falciparum*. One of these compounds, N3, had an IC₅₀ against cultured *P. falciparum* in the nM range, disrupted mitochondrial membrane potential, and had low toxicity against human cells suggesting potential as a lead compound for the development of new anti-malarial agents.

Methods

Chemistry

The hydroxynaphthoquinones N1-5 were recently synthesized using a new methodology [15].

In vitro culture of *P. falciparum*

3D7 strain parasites were cultured and synchronized as described previously [16,17]. Briefly, parasites were routinely maintained in A⁺ human erythrocytes (1-3% parasitaemia and 2% haematocrit) in RPMI-1640 media supplemented with 0.2% sodium bicarbonate, 50 mg/L hypoxanthine and 10% type A+ human serum in 92% N₂, 5% CO₂ and 3% O₂.

Cell culture of HEK293T

HEK293T (human embryonic kidney) cells were cultured in 75 cm² vented tissue culture flasks at 37°C in a humidified atmosphere containing 5% CO₂ in Dulbecco's modified essential medium (Gibco BRL) supplemented with 10% (v/v) foetal bovine serum, 100 U/ml penicillin/ and 100 µg/ml streptomycin.

Flow cytometry analysis

Infected erythrocytes at the ring stage were incubated with different concentrations of the test compounds (N1-N5 0.005, 0.015, 0.045, 0.137, 0.411, 1.23, 3.70, 11.11, 33.33 and 100 µM and atovaquone 0.005, 0.015, 0.045, 0.137, 0.411, 1.23, 3.70, 11.11, 33.33 and 100 nM) for 48 hours; fixed in 2% paraformaldehyde in phosphate-buffered saline (PBS) for 24 hours; permeabilized with 0.1% Triton X-100 and 20 µg/ml RNase; incubated for 30 minutes at 37°C; and stained with 1 nM Yoyo-1 (Molecular Probes). Parasitaemia was determined from dot plots (side scatter versus fluorescence) of 5x10⁴ cells acquired on a FACSCalibur flow cytometer using CELLQUEST software (Becton Dickinson). Initial gating was carried out with unstained, uninfected erythrocytes to account for erythrocyte autofluorescence and analysis performed using Flow Jo 7.6.5 (TreeStar Inc).

Changes in mitochondrial membrane potential ($\Psi\Delta_{mit}$)

Loss of parasite mitochondrial membrane potential ($\Psi\Delta_{mit}$) was determined using 5 µM Mitotracker Red CMXRos as described previously [18]. Cultures were incubated for 30 min at 37°C with the dye and then for 1 h with 10-fold serial dilutions (0.001-100 µM) of N3 and atovaquone. As a control, 5 µM cyanide *m*-chlorophenylhydrazone (CCCP), a protonophore that dissipate the membrane potential, was used. Results were analysed by flow cytometry as described above.

Cytotoxicity assays

The toxicity of hydroxynaphthoquinone derivatives toward HEK293T cells was evaluated with the 4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay [19]. Cells (1.0 × 10⁵/well) were seeded into 48 well plates and incubated in complete medium (Dulbecco's Modified Eagle Media, GIBCO, Life Technologies; supplemented with 10% foetal bovine serum, 100 µg/ml streptomycin and 100 U/ml penicillin) for 24 h. Thereafter, medium was removed and replaced with complete medium (450 µl/well); N3, atovaquone, and solvent (for controls) were added (0.128, 0.64, 3.2, 16, 80 and 400 µM) and cultures were incubated for 48 hours. Cells were then incubated with the MTT reagent for 3 hours, and absorbance was evaluated.

Statistical analyses

Analyses of parasitaemia were performed by a one-way analysis of variance test followed by post hoc analysis by the Dunnett's Multiple Comparison Test using GraphPad Prism software. IC₅₀ values were produced using sigmoid dose-response curves on GraphPad software. At least three independent experiments were performed for each assay.

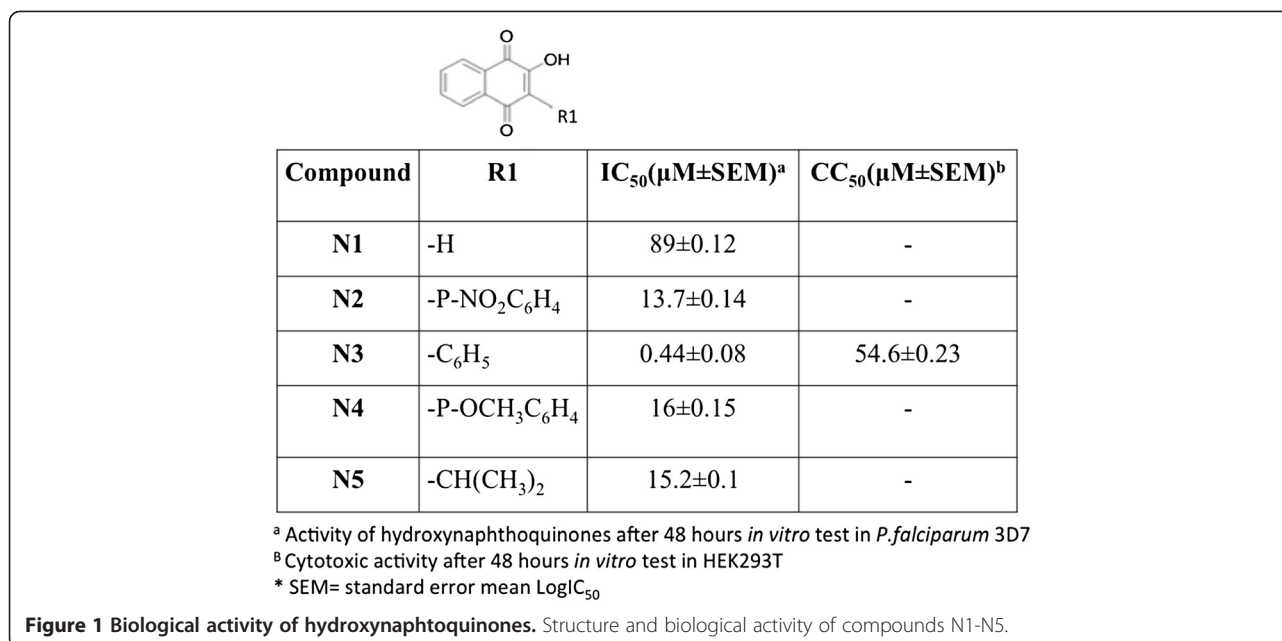
Results

In vitro activity of new hydroxynaphthoquinones

The ability of compounds N1-N5 to disrupt the *in vitro* growth of *P. falciparum* was tested. The naphthoquinones showed activity against *P. falciparum*, with IC₅₀s of 0.4-89 µM (Figure 1 and Additional file 1: Figure S1). Only N3 had an IC₅₀ in the nM range (443 nM; Figure 2).

Cytotoxicity effects on cells HEK293T

Cytotoxic activity against HEK293T cells was assessed with a tetrazolium-based colorimetric assay. No significant cytotoxicity was observed at concentrations below 16 µM. For N3, the concentration leading to 50% cell death (CC₅₀) was 54.6 ± 0.23 µM (Figure 1). For atovaquone the CC₅₀ was 49 ± 0.45 µM.



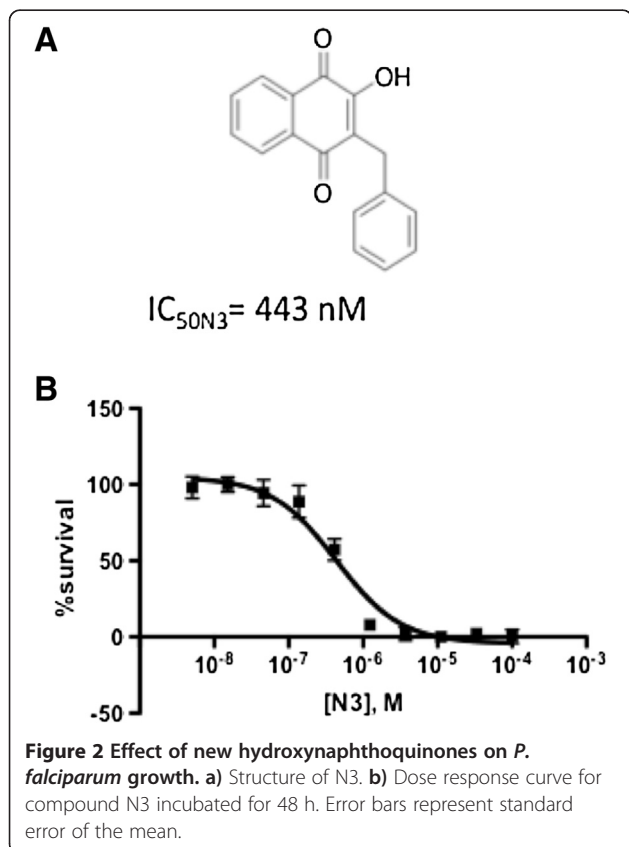
Effect of N3 on ΔΨ_{mit}

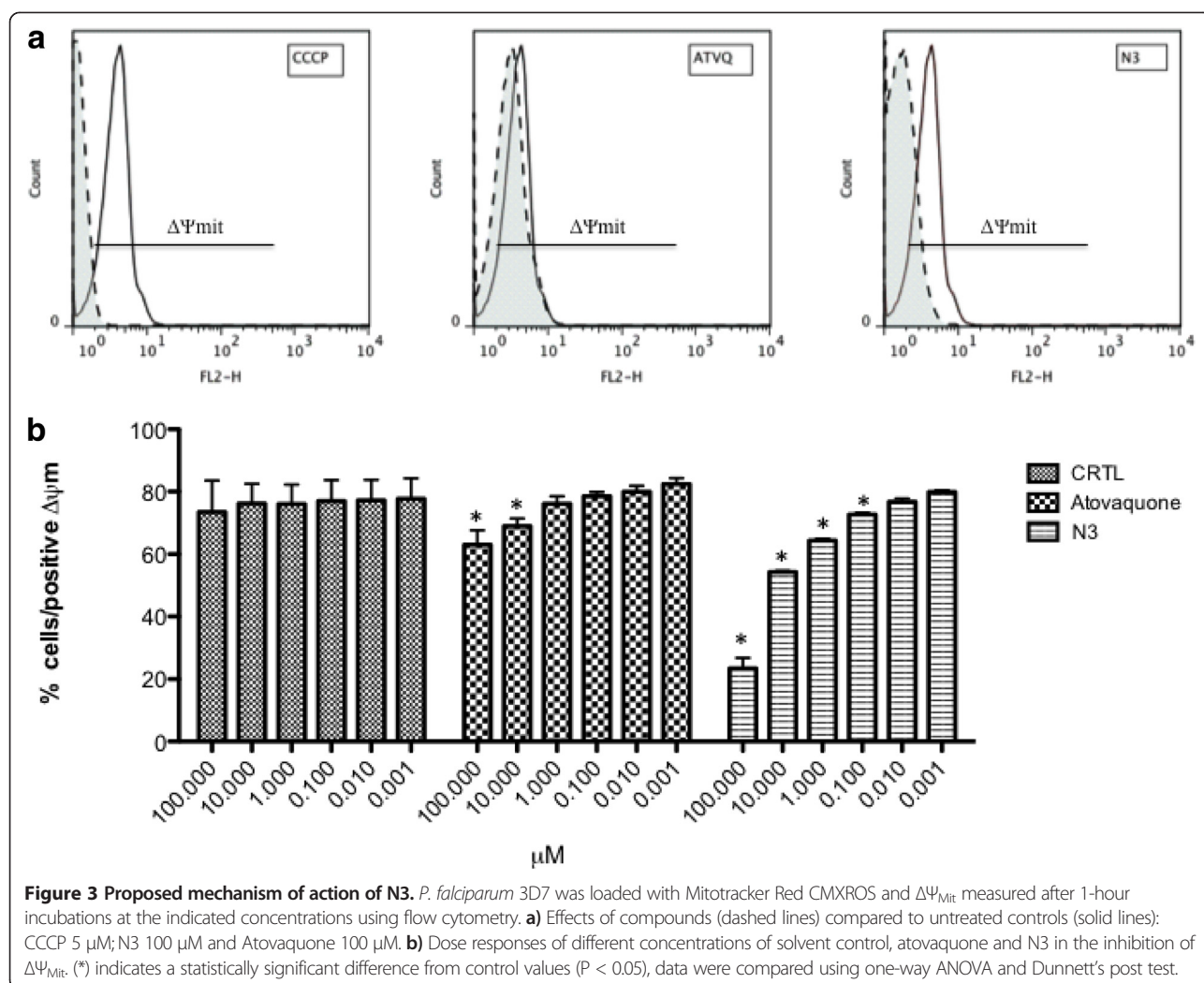
It was also verified the effects of compound N3 on *P. falciparum* mitochondrial membrane potential (ΔΨ_{mit}). Compound N3 showed an IC₅₀ΔΨ_{mit} = 16 μM and atovaquone an IC₅₀ΔΨ_{mit} = 4.4 μM (Figure 3).

Discussion

In an attempt to identify improved anti-malarials, the anti-parasitic activities of synthetic hydroxynaphthoquinones using *in vitro* assays was evaluated. It was identified one compound, N3, with nanomolar activity against *P. falciparum*, confirmed activity against mitochondrial electron transport, and showed limited cytotoxicity against human cells.

The cytochrome bc1 complex catalyses transfer of electrons to maintain the membrane potential of mitochondria, and it is a validated target for anti-malarial drugs. Atovaquone is the only hydroxynaphthoquinone and inhibitor of the bc1 complex currently used to treat malaria. It is generally efficacious, but suffers from irregular absorption (improved with fatty food), limited drug resistance, and high cost of production [8,9,20]. Work to counteract atovaquone limitations has identified other hydroxynaphthoquinones with anti-malarial activity [8]. One series contained an ester at the 3-hydroxy group of atovaquone, with nanomolar anti-malarial activity; addition of long side chains decreased activity [11]. A series of 26 compounds based on the structure of rhinacanthin, a naphthoquinone with anticancer properties, was synthesized [21]; two of these had nanomolar activity and inhibited the cytochrome bc1 complex of *P. falciparum*. Another four hydroxynaphthoquinones were synthesized in an attempt to circumvent resistance to atovaquone, which is mediated by mutations in the mitochondrial cytochrome b gene [13]. The addition of a methyl radical on the naphthoquinone ring provided excellent activity against atovaquone-resistant strains of *P. falciparum*, with documentation of inhibition of the cytochrome bc1 complex [13]. It was recently screened 36 new anti-malarial

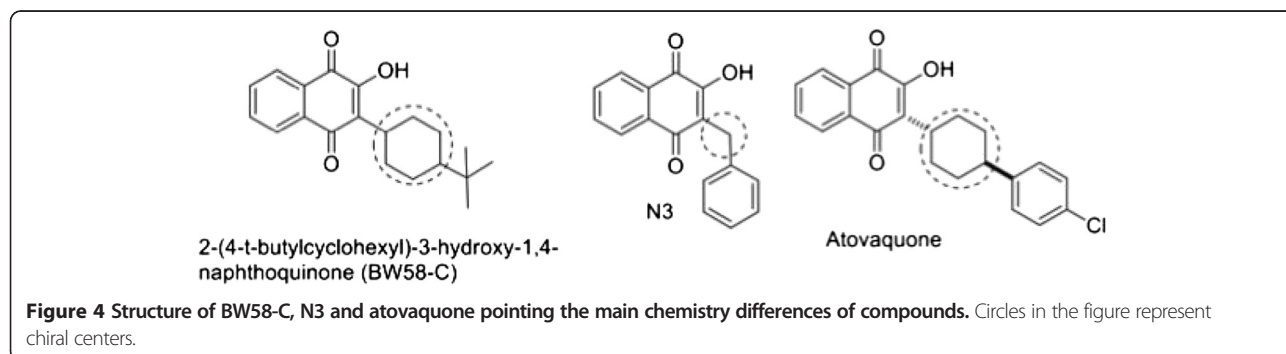




phenylsulfanylmethyl naphthoquinones structurally related to lapachol [22]. The compounds had moderate *in vitro* activity against *P. falciparum*.

Comparing the structures of atovaquone, N3 and BW58-C (an atovaquone precursor), these three structures are very similar in molecular volume, though N3 is much simpler to prepare and has no chiral centers (Figure 4) and, therefore, it can serve as a starting

point for a new series of hydroxynaphthoquinone anti-malarials. The results indicate that the cyclohexane ring of atovaquone is not essential for antimalarial activity, since its replacement by a CH₂ group in N3 only slightly decreased activity, and N3 was capable of inhibiting mitochondrial activity efficiently (Figures 2 and 3). Considering BW58-C, this molecule showed excellent results against murine malaria [23] and good



activity against respiration of mitochondria [24], but it was rapidly metabolized and eliminated in humans [25]. Interaction with cytochrome P450 enzymes and other aspects of metabolism are important components of drug design, and evaluation of the metabolism of N3 is needed.

Screening of a library of 2-hydroxy-naphthoquinones found compounds with alkyl side-chains that effectively inhibited the yeast *bc1* complex [26]. In the present study, was evaluated 5 additional hydroxynaphthoquinones, and demonstrated that one of these, N3, was a potent inhibitor of mitochondrial electron transport, had nanomolar activity against cultured *P. falciparum*, and showed minimal cytotoxicity. Optimization of N3 thus offers potential for new candidate compounds to treat and prevent malaria.

Additional file

Additional file 1: Figure S1. Structures of other hydroxynaphthoquinones and effects on *P. falciparum* growth. Different concentrations of compounds were incubated for 48 h with *P. falciparum*. Results are shown as a dose response curve for compound N1, N2, N4 and N5 incubated for 48 h. Error bars represent standard error of the mean.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

DCS carried out the *in vitro* assays and drafted the manuscript. SBF and DRR carried out the chemical synthesis and collaborated in the elaboration of the manuscript. LNC and MN carried out the cytotoxicity test and collaborated in the elaboration of the manuscript. MN carried out the *Plasmodium* culture and collaborated in the elaboration of the manuscript. CRSG and VFF conceived of the study, and participated in its design and coordination and collaborated in the elaboration of the manuscript. All authors read and approved the final manuscript.

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