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Effects of atovaquone and 4-nitrobenzoate on *Plasmodium falciparum* respiration

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Introduction

Although atovaquone is one of the newest antimalarial compounds discovered, resistant parasites have already been reported[1]. Atovaquone mechanism of action is established to be the competition with ubiquinol (UQH_2) for the bc1 union at mitochondrial cytochrome bc1 complex and preventing the parasite from maintaining an oxidized ubiquinone (UQ) pool, essential for the DHODH activity and consequently for the pyrimidine's biosynthesis. In this sense, possible inhibitors of the ubiquinone biosynthesis pathway would be candidates by stimulating the effects of atovaquone. 4-nitrobenzoate (4-NB) is a well-known inhibitor of 4HPT (4-hydroxybenzoate polyprenyltransferase), the first enzyme of UQ biosynthesis. 4-NB also showed an important effect on reducing the UQs pool in *P. falciparum*. Herein is presenting the effect of atovaquone and 4-NB on parasitic respiration UQ biosynthesis. The purpose of this study was to better understand the atovaquone mechanism of action in a molecular scale, drug target potential of UQ biosynthesis. Oxygen consumption assays revealed 4-NB potentiates atovaquone mitochondrial effects and showed itself the ability to decrease the respiration rate.

Materials and Methods

Plasmodium falciparum asexual stages culture. P. falciparum 3D7 strain was cultured *in vitro* by Trager and Jensen methodology[2] using RPMI-1640 medium complemented with Albumax I (0.5%) (Thermo Fisher Scientific) and a gaseous mixture of 5% CO_2, 5% O_2, and 90% N_2 was employed as a reference. However, for some experiments it was used oxygen-free mixtures (5% CO_2 and 95% N_2)[3] (Supplemental data, figure S1). All gaseous mixtures were purchased from Air Products Brasil LTDA (São Paulo, SP, Brazil). Culture synchronization was performed by 5% (w/v) D-sorbitol solution as previously described by C. Lambros & J.P. Vanderbergand[4] parasitic stages and parasitemia were monitored by Giemsa-stained smears microscopy.

Oxygen consumption assays. Oxygen consumption assays were performed based on Murphy et al previous studies[5]. For this purpose all experiments were performed three times by employing the same amounts of parasites ($1x10^8$ cells). Briefly, treated/untreated cultures at schizont stages were centrifuged at 1500 x *g* for 5 min at 4 °C and lysed with 30 mL 0.03 % saponin in PBS at 25 ºC for 5 min. Parasites were then centrifuged 1500 x *g* for 5 min at 4 °C and subsequently washed in Respiration Buffer (125 mM sucrose, 65 mM KCl, 10 mM Hepes-KOH, pH 7.2, 5 mM $MgCl_2$, 2 mM KH_2PO_4, 0.5 mM EGTA). Parasites were resuspended at $1x10^9$ cells/mL in the same culture medium and oxygen uptake was measured immedi ately. The oxygen consumption assay was performed in 2 ml at ºC 37°C in a high-resolution oxygraph (Oxygraph-2k Oroborus Instruments, Innsbruck, Austria). Drug additions while the oxygen uptake assay or 48h treatments during parasitic culture.

Results

The routine respiration in isolated schizonts provided by the oxygen consumption assays, allowed a better understanding of the 4-NB effects on mitochondrial activity. 4-NB pre-treated parasites showed reduced oxygen consumption at 1 mM, in which the compound has low effect on parasite growth. The study of 4-NB antiplasmodial effect revealed that the compound has an inhibitory effect only at milimolar concentrations. (20% growth decrease at 1 mM; IC_{50} = 2.56 ± 0.12). Curiously, 50 µM 4-NB pre-treated parasites do not induce a decrease in routine respiration when compared with untreated parasites (Fig 1A and B) but the parasites were more susceptible to atovaquone (Fig 1C). Was also found that 4-NB inhibit the oxygen consumption above 100 µM in a dose-dependent response, when measured immediately after the incubation (Fig 1D).

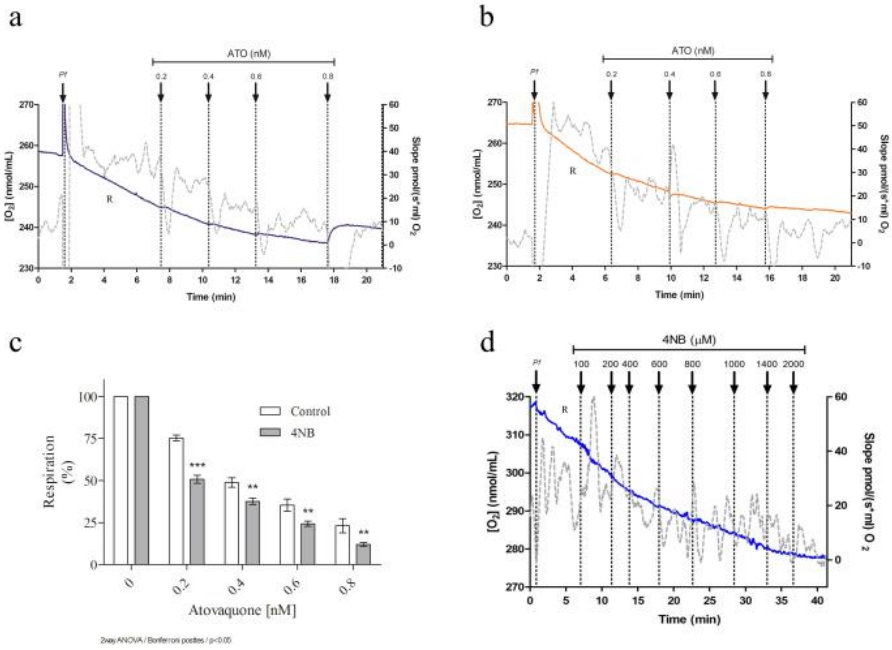

Fig 1. Respiratory rates in isolated esquizonts. Oxygen consumption was measured after saponization. The gray lines indicate the slope variation of oxygen concentration as a function of time (right axis). The blue and orange lines represent O_2 concentration (left axis). (A) as a positive control the parasites were maintained in respiratory buffer. (B) Cultures were treated with 4-NB before the saponization. ATO, atovaquone at diferent concentrations was added in both cases. R: routine respiration. (C) Respiration percentage; the respiration slopes under particular atovaquone concentrations were normalized by the corresponding routine slope. (D) Cultures were treated with progressive 4-NB concentrations, after the saponization and throughout the oxygen measurement. One-way ANOVA followed by a Tukey's posttest was used for statistical analysis to compare the values to the respective control. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$ (Tukey's post-test).

Discussion

The increase of atovaquone effect on respiration by 4-NB treatment could be explained by the already described action mechanism of 4-NB. This compound was studied by Forsman et al[6] and was verified the capacity to inhibit the first enzyme of Ubiquinone biosynthesis. At higher concentrations (above 100 µM) 4-NB could be performing another mechanism of action, directly in mitochondria. Further studies will be needed to understand this phenomenon. The results exposed here highlight to an unexplored possibility of synergistic interaction between 4-NB and atovaquone for malaria treatment.

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