Type II NADH Dehydrogenase Inhibitor 1-Hydroxy-2-Dodecyl-4(1H)Quinolone Leads to Collapse of Mitochondrial Inner-Membrane Potential and ATP Depletion in Toxoplasma gondii

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The apicomplexan parasite Toxoplasma gondii expresses type II NADH dehydrogenases (NDH2s) instead of canonical complex I at the inner mitochondrial membrane. These non-proton-pumping enzymes are considered to be promising drug targets due to their absence in mammalian cells. We recently showed by inhibition kinetics that T. gondii NDH2-1 is a target of the quinolone-like compound 1-hydroxy-2-dodecyl-4(1H)quinolone (HDQ), which inhibits T. gondii replication in the nanomolar range. In this study, the cationic fluorescent probes Mitotracker and DiOC6(3) (3,3′-dihexyloxycarbocyanine iodine) were used to monitor the influence of HDQ on the mitochondrial inner membrane potential (∆Ψm) in T. gondii. Real-time imaging revealed that nanomolar HDQ concentrations led to a ∆Ψm collapse within minutes, which is followed by severe ATP depletions of 30% after 1 h and 70% after 24 h. ∆Ψm depolarization was attenuated when substrates for other dehydrogenases that can donate electrons to ubiquinone were added to digitonin-permeabilized cells or when infected cultures were treated with the F,–ATPase inhibitor oligomycin. A prolonged treatment with sublethal concentrations of HDQ induced differentiation into bradyzoites. This dormant stage is likely to be less dependent on the ∆Ψm, since ∆Ψm-positive parasites were found at a significantly lower frequency in alkaline-pH-induced bradyzoites than in tachyzoites. Together, our studies reveal that oxidative phosphorylation is essential for maintaining the ATP level in the fast-growing tachyzoite stage and that HDQ interferes with this pathway by inhibiting the electron transport chain at the level of ubiquinone reduction.

The apicomplexan parasite Toxoplasma gondii contains a single mitochondrion of an elongated tubular structure (28, 32), which shows several significant metabolic differences from the mammalian counterpart (see references 24 and 33 for review). Although the T. gondii mitochondrion harbors the complete set of enzymes for the tricarboxylic acid cycle (15), it lacks the pyruvate dehydrogenase complex (7, 14, 18), which is typically a central enzyme in carbohydrate metabolism that catalyzes the decarboxylation from pyruvate to acetyl coenzyme A. Other mitochondrial pathways for mitochondrial acetyl coenzyme A generation, such as the 2-methylcitrate cycle, are currently under investigation (33). The T. gondii genome predicts the presence of all components necessary for a respiratory chain. Biochemical evidence for oxidative phosphorylation was provided by extracellular T. gondii tachyzoites that were permeabilized with digitonin (39). However, the overall contribution of oxidative phosphorylation to energy production in relation to other ATP-generating pathways has not been satisfactorily clarified for intracellular T. gondii so far.

A fundamental difference of the T. gondii and also the Plasmodium falciparum electron transport chains (ETCs) as opposed to the mammalian ETC is the lack of multisubunit complex I, which couples the transfer of electrons from NADH to ubiquinone with the translocation of protons (6). Instead, P. falciparum expresses one isoform (2) and T. gondii expresses two isoforms (22) of so-called “alternative” or type II NADH dehydrogenases (NDH2s). These single-subunit enzymes do not transport protons across the membrane, and they are, in contrast to the NADH-oxidizing activity of complex I, not rotenone sensitive (21, 27). NDH2s can occur in two topological orientations with respect to the inner mitochondrial membrane. Internal enzymes are facing with their active site toward the mitochondrial matrix and use mitochondrial NAD(P)H as the electron donor, while external enzymes use cytosolic NAD(P)H. Up to now, the orientation of the apicomplexan isoforms is unknown.

Due to their absence in the mammalian host, NDH2s were proposed to be promising drug targets against Mycobacterium tuberculosis (40). Their suitability as a drug target in Plasmodium is controversial and has been the subject of discussion (16, 17, 38). Previously, it was demonstrated that low-affinity NDH2 inhibitors in micromolar concentrations were able to inhibit the activity of the P. falciparum NDH2 and led to a collapse of the mitochondrial membrane potential (ΔΨm) (2). The only high-affinity NDH2 inhibitors described so far are 1-hydroxy-2-alkyl-4(1H)quinolones with long alkyl-site chains, for example, 1-hydroxy-2-dodecyl-4(1H)quinolone (HDO) (C12), which possesses structural similarity to ubiquinone. These compounds were shown to inhibit the activities of Yarrowia lipolytica NDH2 (13) and T. gondii NDH2-I (22) with 50% inhibitory concentrations of between 200 and 300 nM. HDO was also shown to effectively inhibit T. gondii and P. falciparum NDH2.
falciparum replication in nanomolar concentrations in tissue cultures (31). We demonstrate in this study that HDQ treatment in nanomolar concentrations leads to a depolarization of the T. gondii ΔΨm within minutes. The subsequent lack of oxidative phosphorylation leads to a ~70% reduction of the intracellular ATP level within 24 h. This suggests an indispensable role of NDH2 activity in the maintenance of the ΔΨm and in energy metabolism in the tachyzoite stage.

MATERIALS AND METHODS

T. gondii cultivation, in vitro stage conversion, and cell lines. Tachyzoites were propagated in human foreskin fibroblasts (HFFs) as previously described (30). Bradyzoite differentiation was induced by an alkaline-pH shift (34). In brief, tachyzoites were freshly released by syringe passage, and 3×10^6 parasites were inoculated onto a confluent HFF monolayer grown on a 24-well imaging plate. The pH shift medium (pH 8.3) was exchanged daily to maintain a constant pH. 143B/206 cells, which lack mitochondrial DNA (20), and the parental 143B cell line were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 μg/ml), 1% glutamine, sodium pyruvate (110 μg/ml), and uridine (50 μg/ml).

Chemicals. HDQ was kindly provided by W. Oettmeier and was dissolved in dimethyl sulfoxide or ethanol. Oligomycin, atovaquone, tetramethylp-phenylenediamine (TMPD), ascorbate, malate, succinate, glyceraldehyde-phosphate, dehydoroacetate, oxaloacetate, uracil, and digitin were purchased from Sigma.

Plasmids. Plasmid sub-S9-RFP/sag-CAT was constructed by replacing the FNR fragment from BglII/AvrII-digested sub-FNR-RFP/CAT (36) with a BglII/AvrII S9 fragment from sub5-S9-GFP/CAT (8). For generating pTet7Sag–TgATP-β-cmyc-DHFR, the complete open reading frame (ORF) of the T. gondii ATPase β subunit (TgATP-β) (GenBank accession number DQ228960) was amplified from RH cDNA using Phusion polymerase (Promega) with the primer set consisting of primers 5′-TAATGCATAAAATGGCGTCCTCCG CACTC (NsiI) and 5′-TACCTAGGTTCCTGCGCCCTTCTG (AvrII). The PCR fragment was cloned into pCR4-TOPO (Invitrogen), and the DNA was sequenced. Finally, the NsiI/AvrII fragment was subcloned into vector pTet7Sag–ACP-cmyc-DHFR (kindly provided by B. Striepen), thereby replacing the acyl carrier protein ORF with the ATP-β ORF. The final construct consists of the anhydrotyrosine-regulatable TetO7Sag4 promoter element (25), which controls expression of the complete TgATPase-β ORF with a C-terminal myc tag, and additionally includes a pyrimethamine resistance cassette for selection (9).

Generation of transgenic parasites. The transfection of T. gondii strain RH TAT1 (26) was carried out based on previously reported protocols (30, 35). Stable transgenic parasite lines expressing TgATP-β and S9-red fluorescent protein (RFP) were selected with 1 μM pyrimethamine and 20 μM chloramphenicol, respectively.

Detection of ΔΨm in T. gondii. The T. gondii ΔΨm was monitored after staining with the fluorochrome Mitotracker or DiOC6(3) (3,3′-dihexyloxacarboxyanine iodide; Invitrogen). Both fluorescent dyes were dissolved in tissue-culture-grade dimethyl sulfoxide. For Mitotracker, the staining solution was prepared with the same procedures by using a fluorescein isothiocyanate-conjugated lectin from Dolichos biflorus (1:300; Sigma) for 1 h each. To detect bradyzoites, a bryozoyte-specific anti-BAG1 MAb (7E5) (3) and a Cy3-conjugated anti-mouse IgG antibody (1:300; Dianova) were used instead. Lein staining was performed with the same procedures by using a fluorescent isothiocyanate-conjugated lectin from Dolichos biflorus (1:300; Sigma).

RNA extraction and real-time PCR. Total RNA was isolated using the GenElute mammalian total RNA kit (Sigma), and reverse transcription was done using 5 μg of total RNA, oligo(dT) primer (Sigma), and Molloney murine leukaemia virus reverse transcriptase (RNase H minus; Promega) according to the manufacturer’s instructions. Real-time PCR was performed using LightCycler PCR (Roche) to amplify cDNA for the mRNA transcript levels of the bradyzoite-specific genes enolase 1 and bag1 as well as β-tubulin. The primer sets used for cDNA amplification were 5′-CGAGGGGTGCTGAAAAGATATTCC-3′ and 5′-CAGGGAGGCCACCAAGAAGTG-3′ for enolase 1, 5′-GACCACATGCTCTTAAACAGC-3′ and 5′-CCGCAACCAGGGCCCTACCTAG-3′ for bag1, and 5′-CCGCAACCAGGGCCCTACCTAG-3′ and 5′-TAAGCGCTCCCTTCTG CACCC-3′ for β-tubulin, respectively. PCR amplification was carried out using the following parameters: 10 min at 95°C followed by 40 cycles of denaturation (95°C for 10 s), annealing (60°C for 5 s), and extension (72°C for 15 s). All samples were tested for PCR amplification efficiencies according to manufacturer’s protocols and software (Roche). Serial dilutions of cDNAs were subjected to real-time PCR amplification in duplicates using the β-tubulin primer pair. Crossing points were plotted against the log of cDNA dilutions, and amplification efficiencies (E) were calculated from the slopes of the obtained lines by the following formula: E = 10^(-1/slope). The difference of amplification efficiencies (ΔE) of HDQ-treated samples and the untreated control was less than 0.05 (ΔE_HDQ_100 nM - control = 0.019; ΔE_HDQ_1000 nM - control = 0.013), and crossing-point values from PCR amplification could thus be used for relative quantification. β-Tubulin mRNA levels were used for the normalization of enolase 1 and bag1 transcript levels in HDQ-treated and -untreated samples.

Protein fractionation and immunoblotting. Protein extracts were prepared at 4°C throughout. Extracellular tachyzoites (3×10^7 to 4×10^7 tachyzoites) from a stable transgenic line expressing TgATPase-β were harvested and resuspended in ice-cold PBS containing protease inhibitors. Parasites were lysed by repetitive
freeze-and-thaw steps and sonicated five times for 10 s each. Subsequently, the lysates were separated after centrifugation at 13,000 × g for 45 min. The supernatant comprising the membrane-soluble fraction was collected, precipitated by trichloroacetic acid, and resuspended in 2× sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer containing 0.09 M Tris-Cl (pH 6.8), 20% glycerol, 2% SDS, 0.02% bromophenol blue, and 0.1 M dithiothreitol. The pellet fraction was also resuspended in the same buffer. Protein fractions were resolved on an SDS-polyacrylamide gel and electroblotted onto a Hybond nitrocellulose membrane (Amersham Biosciences). Bound proteins were probed with an anti-myc MAb (1:500), followed by a secondary antibody with goat anti-mouse IgG coupled to alkaline phosphatase (1:2,000; Dianova). Reactive proteins were detected by alkaline phosphatase staining solution containing 0.05% bromo-4-chloro-3-indolyl phosphate and 0.5% nitroblue tetrazolium (Sigma) as substrates.

RESULTS

HDQ treatment leads to a collapse of the ΔΨm. The influence of the high-affinity NDH2 inhibitor HDQ (13, 22) on the ΔΨm was investigated with the aid of the cationic fluorescent dye Mitotracker. When mock controls were stained with Mitotracker at different time points from 7 to 32 h postinfection, 75 to 80% of all intracellular parasites showed the typical intense staining of the single T. gondii mitochondrion (Fig. 1A) and were thus categorized as being ΔΨm positive. The frequencies of ΔΨm-positive parasites were similar in all samples and unrelated to the size of the parasitophorous vacuoles, suggesting that the majority of tachyzoites possess a ΔΨm throughout their intracellular life span. In contrast, mitochondrial staining was absent in more than 85% of the parasites when cultures were incubated with 100 nM HDQ (Fig. 1A and B) for the last 6 h. In the remaining parasites, which were classified as being ΔΨm positive, the intensity of the staining appeared to be less than that for the untreated controls. This suggests that HDQ treatment leads to a collapse of the ΔΨm. Furthermore, it indicates that the susceptibility to membrane depolarization is independent from the size of the parasitophorous vacuole.

Real-time monitoring of the T. gondii ΔΨm. Mitotracker comprises a fluorophore moiety that forms covalent bonds with the SH groups of mitochondrial membrane proteins, which allows the fixation of the stained samples before microscopic analysis. A drawback of this feature is that Mitotracker cannot be reliably used to monitor changes in the ΔΨm in living cells, since the thiol bond formation is nonreversible. In order to determine the kinetics of an HDQ-mediated ΔΨm collapse in intracellular T. gondii, we first searched for a ΔΨm-sensitive dye that allows real-time imaging of the T. gondii ΔΨm. We compared the cationic fluorophores TMRE (tetramethylrhodamine ethyl ester), JC-1 (3,3′,4,4′-tetrakis(4-sulfonic acid)-4′,3′-diethylrhodamine), and DiOC6(3) for their abilities to specifically stain the mitochondrion of intracellular tachyzoites with no or only weak staining of the plasma membrane. DiOC6(3) was found to be the most suitable dye for this purpose, since it resulted in an intense and specific mitochondrial staining pattern, which perfectly colocalized with a mitochondrially targeted RFP (S9-RFP) (Fig. 2A). When intracellular parasites were treated with 10 nM of the well-established complex III inhibitor atovaquone (1), the intensity of the DiOC6(3) staining decreased gradually over time, leading to a complete depolarization of the ΔΨm within 40 min in >60% of the parasites (Fig. 2B and C). These results are consistent with previously reported observations of the mitochondrial membrane depolarization effect of atovaquone in T. gondii (39) and thus demonstrate the suitability of DiOC6(3) staining for real-time ΔΨm imaging. The dye could be used for up to 1.5 h in real-time imaging, with a maximum of 10 to 15 exposures taken within this time. Higher numbers of exposures resulted in a significant bleaching effect, and at incubation times longer than 1.5 h, the dye showed a tendency to lose the equal distribution across the mitochondrial membrane and started to accumulate at a single location. We used DiOC6(3)-based real-time imaging in the following experiments to monitor the kinetics of HDQ-mediated ΔΨm depolarization.

Kinetics of HDQ-mediated ΔΨm collapse. DiOC6(3)-based real-time imaging of the ΔΨm for parasites treated with 10
nM, 100 nM, and 1 μM HDQ led to a dose-dependent depolarization of the T. gondii mitochondrial membrane (Fig. 2B and C). At 1 μM HDQ, the mitochondrial membrane from more than 75% of the parasites was completely depolarized within the first 5 min, demonstrating a fast mode of action of the drug (Fig. 2C). The depolarization kinetics of 10 nM HDQ were similar to those of 10 nM atovaquone. A combination of 10 nM HDQ with 10 nM atovaquone resulted in a significantly faster collapse of the ΔΨm than treatment with 10 nM of the individual drugs, which is in agreement with a synergistic mode of action between HDQ and atovaquone (31).

**Substrates for ubiquinone-reducing enzymes lead to ΔΨm stabilization.** Aside from the two type II NADH dehydrogenases, T. gondii possesses a further four enzymes that can feed electrons into the ubiquinol pool, namely, succinate dehydrogenase, malate:quinone oxidoreductase, dihydroorotate dehydrogenase (DHODH), and glycerol-3-phosphate dehydrogenase. We investigated whether an excess of substrates for these enzymes could compensate for an HDQ-mediated depolarization of the ΔΨm. Cells were treated for this purpose with 2 μM digitonin, a concentration which was shown previously to selectively permeabilize the parasite’s plasma membrane for metabolites without disturbing the function of the respiratory chain (39). The ΔΨm of living, intracellular parasites was monitored by DiOC6(3) staining. In drug-untreated controls, more than 80% of the parasites displayed a strong ΔΨm, confirming that the digitonin treatment itself did not affect the ΔΨm (Fig. 3). Treatment with TMPD-ascorbate, a combination which is commonly used to feed electrons into complex IV, led to a strongly attenuated ΔΨm depolarization after HDQ treatment, indicating that HDQ inhibits the ETC upstream of complex IV. The addition of dihydroorotate, glycerol-3-phosphate, malate, or succinate did not prevent an atovaquone-mediated ΔΨm collapse. This was the expected result, since atovaquone, as a complex III inhibitor, blocks the ETC downstream of ubiquinone reduction. However, each of the four substrates significantly increased the number of ΔΨm-positive parasites in the presence of HDQ (Fig. 3). The highest number of ΔΨm-positive parasites in HDQ-treated cultures was achieved when all four substrates were added simultaneously. Oxalacete was used as a control and did not result in an increased frequency of ΔΨm-positive parasites. Together, these results suggest that HDQ possesses a different mode of action compared to that of atovaquone and inhibits the ETC upstream of complex III in the level of ubiquinone reduction.

**Oligomycin-mediated inhibition of the T. gondii F$_0$F$_1$-ATPase attenuates HDQ-mediated ΔΨm depolarization.** We next investigated whether the ΔΨm can be stabilized in HDQ-treated parasites by an inhibition of the putative T. gondii F$_0$F$_1$-ATPase. The inner mitochondrial membrane is impermeable to protons, and the only possibility for protons to reenter the mitochondrial matrix is through the F$_1$ proton channel, which can be inhibited by oligomycin. The T. gondii genome contains all genes for the five parts (α, β, γ, δ, and ε) that form the F$_1$ subunit. However, the parasite appears to possess an unusual F$_0$ subunit, since from the three proteins (F$_0$-a, F$_0$-b, and F$_0$-c) that typically form the F$_0$ subunit, obvious homologues for F$_0$-a and F$_0$-b are lacking (23). This indicates either a high degree of divergence in the lacking parts or an unusual composition of the F$_0$ subunit. In Plasmodium, which is lacking all three F$_0$-forming proteins, a matrix localization of the F$_1$ subunit was previously proposed, which implies that the proton gradient cannot be used for ATP synthesis (29). In order to discriminate between a matrix and a membrane association of the F$_1$ subunit, we examined the localization of the F$_1$-ATPase in parasites, which expressed an epitope-tagged version of ATPase-β (TgATP-β), which is a part of the F$_1$-ATPase. Myc-tagged TgATP-β of stably transfected parasites was targeted to the mitochondrion, as shown by the colocalization with the Mitotracker signal (Fig. 4A). After fractionation of the T. gondii lysate, ATPase-β was found exclusively in the membrane fraction and was absent in the soluble fraction, suggesting that T. gondii possesses a typical, membrane-associated mitochondrial F$_1$-ATPase (Fig. 4B).

The effect of oligomycin-mediated F$_0$F$_1$-ATPase inhibition was further investigated by using 143B/260 cells as host cells for T. gondii. This cell line lacks a functional mitochondrial respiratory chain (20), which excludes the possibility that oligomycin has an indirect effect on T. gondii via an inhibition of host cell ATP synthesis. The fraction of parasites with positive Mitotracker staining was determined using 143B/260 host cells after HDQ treatment with and without the addition of oligomycin. Oligomycin treatment resulted in a strong increase in levels of ΔΨm-positive parasites (Fig. 4C), suggesting that the HDQ-mediated ΔΨm depolarization can be attenuated by preventing protons from reentering the mitochondrial matrix.

**HDQ treatment leads to a decreased ATP level.** We further examined the influence of HDQ-mediated ΔΨm collapse on the parasitic ATP level. Infected cultures were incubated with 1 μM HDQ, and intracellular parasites were mechanically released from host cells by syringe passage at 1, 3, 8, and 24 h after the addition of HDQ. The ATP level of the harvested parasites was determined using a luminescence assay, and the obtained values were normalized for parasite numbers. An oligomycin-treated sample was included in order to quantify ATP levels in parasites in which F$_0$F$_1$-ATPase activity was.
inhibited. The kinetics revealed that HDQ leads to a gradual decrease of the parasite’s total ATP level, resulting in a ~30% reduction after 1 h and a ~70% reduction after 24 h (Fig. 5).

A 70% decrease in the ATP level was also observed after the complete inhibition of $F_oF_1$-ATPase activity using 1 mM oligomycin.

HDQ growth inhibition is not mediated by pyrimidine starvation. HDQ possesses structural similarities to ubiquinol and

**FIG. 3.** Substrate supplementation in permeabilized parasites partly decreases HDQ-mediated $\Delta\Psi_m$ depolarization. DiOC$_6$(3)-stained intracellular parasites were digitonin permeabilized and treated with 1 mM HDQ (A) or 1 mM atovaquone (ATO) (B) either alone or in combination with 10 mM malate (MAL); 10 mM succinate (SUC); 10 mM dihydroorotate (DHO); 1 mM glycerol-3-phosphate (G-3-P); 10 mM oxaloacetate (OAA); a mixture of malate, succinate, dihydroorotate, and glycerol-3-phosphate (SUB); and 0.2 mM TMPD–1.5 mM ascorbate (TMPD/ASC). The percentage of $\Delta\Psi_m$-positive parasites was determined from pictures taken by fluorescence microscopy after a 15- to 25-min incubation period at 37°C. Results are expressed as means ± SD of data from duplicate samples from a representative experiment ($n=2$). * , $P<0.002$; **, $P<0.003$; ***, $P<0.005$; ****, $P<0.003$; ##, $P<0.001$ (determined by a Student’s $t$ test) (A). * , $P<0.003$ (determined by a Student’s $t$ test) (B).
is believed to interact with the ubiquinol binding site of type II NADH dehydrogenases (13). One of the enzymes also possessing a ubiquinol binding site is DHODH, which catalyzes the fourth step in de novo pyrimidine biosynthesis. Since *P. falciparum* DHODH was recently described to be inhibited by HDQ (10), we examined whether HDQ exerts its growth-inhibitory effect on *T. gondii* by pyrimidine starvation.

*T. gondii* possesses a pyrimidine salvage pathway, and parasites deficient in de novo pyrimidine synthesis can be rescued with high concentrations of uracil, which is converted by the parasitic uracil-phosphoribosyltransferase to UMP (19). We supplemented the culture medium with 250 μM uracil and determined the *T. gondii* growth rate in the presence of 100 nM HDQ. Uracil supplementation did not lead to an increased growth rate (Fig. 6), suggesting that the underlying mechanism of HDQ growth inhibition is not due to pyrimidine starvation.

**HDQ induces bradyzoite differentiation.** HDQ was shown to effectively inhibit parasite replication (31). Since a reduction in the level of parasite replication in *T. gondii* is often associated with stage differentiation (4), we investigated whether HDQ treatment leads to bradyzoite differentiation. HFFs were infected with tachyzoites and cultivated in the presence of 100 nM and 1 μM HDQ for 72 h. Subsequently, the transcript levels of the bradyzoite marker genes *bag1* (5) and enolase 1 (11) were determined by real-time PCR, in comparison to drug-untreated controls. Both genes were upregulated between six- and ninefold after HDQ treatment (Fig. 7), indicating that HDQ leads to a moderate induction of bradyzoite differentiation.

**FIG. 4.** Inhibition of membrane-associated *T. gondii* F, F1-ATPase attenuates HDQ-mediated ΔΨm depolarization. (A) RH strain tachyzoites stably transfected with pTet7Sag4-TgATP-β-cmyc-DHFR were analyzed by immunofluorescence assay using anti-myc MAb. Mitochondrial localization of myc-tagged ATPase-β was confirmed by colocalization with Mitotracker fluorescence. (B) Parasites expressing myc-tagged ATPase-β were fractionated into a soluble fraction (S) and a membranous fraction (P). Both fractions were separated by SDS-PAGE and analyzed by immunoblotting with an anti-myc antibody. (C) The mitochondrial DNA-lacking cell line 143B/260 was infected with *T. gondii* RH strain cells and treated at 18 h postinfection with 100 nM HDQ or 1 μM oligomycin for 6 h. Parasites were released from host cells by syringe passage and immediately stained with Mitotracker. The percentage of ΔΨm-positive parasites was determined by fluorescence microscopy after the fixation of at least 150 parasites. The diagram shows the means ± SD of data from duplicates from a representative experiment. ut, untreated. *, P < 0.002; **, P < 0.05 (determined by a Student’s *t* test).

**FIG. 5.** HDQ treatment leads to a decreased ATP level. HFFs were infected with RH strain parasites and, after 24 h, treated with 1 μM HDQ for the indicated time periods or with 1 μM oligomycin for 24 h. Intracellular parasites were released by syringe passages, and the ATP level was quantified as photons per second (CPS) in a luminescence assay. Relative ATP levels were normalized for parasite numbers. Results are expressed as means ± SD of data from duplicate wells from a representative experiment (*n* = 3). *, P < 0.003; **, P < 0.002; ***, P < 0.001 versus untreated control (determined by a Student’s *t* test).

**FIG. 6.** HDQ-mediated growth inhibition is not mediated by pyrimidine starvation. RH strain tachyzoites were treated with 100 nM HDQ in the presence or absence of 250 μM uracil. Duplicate samples were fixed after 24 h, and the average number of tachyzoites per vacuole was determined. At least 100 vacuoles were examined for each sample. Results are represented as means ± SD of data from a representative experiment (*n* = 2).
The frequency of ΔΨm-positive parasites decreases during bradyzoite differentiation. There are several indications that the energy metabolism of bradyzoites is different from that of tachyzoites (12, 41), and it was thus of interest to compare the frequencies of ΔΨm-positive parasites in both stages. Bradyzoite differentiation was induced by an alkaline-pH shift, and cyanate-conjugated tosolic small heat shock protein, and with a fluorescein isothiocyanate-bradyzoite-specific anti-BAG1 antibody, which detects a cytoplasmic BAG1-positive population was less than 10%, compared to 85% after 24 h and to 20% after 72 h, while the expression of the bradyzoite markers bag1 and enolase 1. HFFs were infected with tachyzoites and cultivated in the presence of 100 nM and 1 μM HDQ for 72 h. Enolase 1 and bag1 mRNA transcripts were determined by real-time PCR. β-Tubulin was used for normalization. The diagram shows enolase 1 and bag1 transcript levels of HDQ-treated samples relative to that of a mock-infected control (arbitrarily defined as 1), which was harvested 24 h postinfection. Results are expressed as means ± SD of data from duplicate samples from a representative experiment. *, *P < 0.0001; **, P < 0.0002 versus mock control (determined by a Student’s t test).

**FIG. 7.** HDQ treatment upregulates transcript levels of the bradyzoite markers bag1 and enolase 1. HFFs were infected with tachyzoites and cultivated in the presence of 100 nM and 1 μM HDQ for 72 h. Enolase 1 and bag1 mRNA transcripts were determined by real-time PCR. β-Tubulin was used for normalization. The diagram shows enolase 1 and bag1 transcript levels of HDQ-treated samples relative to that of a mock-infected control (arbitrarily defined as 1), which was harvested 24 h postinfection. Results are expressed as means ± SD of data from duplicate samples from a representative experiment. *, *P < 0.0001; **, P < 0.0002 versus mock control (determined by a Student’s t test).

**DISCUSSION**

We demonstrate in this study that the treatment of intracellular tachyzoites with HDQ, a quinolone-like compound that was previously shown to inhibit TgNDH2-I (22), leads to a fast, dose-dependent collapse of the ΔΨm and subsequently to a decrease in the intracellular ATP level. The mode of action of HDQ in T. gondii is thus an inhibition of oxidative phosphorylation. The observed synergism of 10 nM atovaquone on ΔΨm depolarization is in agreement with the synergism of these drugs to inhibit parasite replication in vitro (31). The addition of succinate, dihydroorotate, or malate to digitonin-permeabilized cells stabilized the ΔΨm in the presence of HDQ, whereas these substrates did not influence atovaquone-mediated depolarization. This observation clearly indicates that HDQ acts as an ETC inhibitor upstream of the atovaquone target, which is complex III, at the level of ubiquinone reduction. The attenuation of HDQ-mediated ΔΨm depolarization in the presence of high substrate concentrations of ubiquinone-reducing enzymes is in agreement with HDQ acting as an NDH2 inhibitor and a replenishment of the ubiquinol pool by an increased level of activity of the corresponding enzymes succinate dehydrogenase, DHODH, glycerol-3-phosphate dehydrogenase, and malate:quinone oxidoreductase. Since physiological substrate concentrations in non-digitonin-treated cells are not sufficient to compensate for the HDQ-mediated ΔΨm depolarization, a major role of NDH2 activity in providing the ETC with reduction equivalents can be assumed under the assumption that HDQ is not affecting any other targets of the ETC. Although we have no indications for potential other targets of HDQ in T. gondii, we cannot completely rule out the possibility that HDQ also exerts an inhibitory effect on one or more of the above-mentioned ubiquinone-reducing enzymes. However, we excluded the possibility that pyrimidine starvation is the mode of action by which HDQ inhibits T. gondii replication. A recent study reported that HDQ inhibits DHODH of P. falciparum (10). This ubiquinone-dependent enzyme catalyzes the dehydrogenation of dihydroorotate to orotate, an essential step for de novo pyrimidine biosynthesis. In contrast to Plasmodium, T. gondii possesses a pyrimidine salvage pathway, and parasites deficient in de novo pyrimidine synthesis can be rescued with high uracil concentrations (19). Since uracil supplementation did not rescue parasite replication in HDQ-treated cultures, we could exclude that pyrimidine starvation is the major mode of inhibition of HDQ in T. gondii.

F0F1-ATPases use the proton motive force across the inner mitochondrial membrane for coupling proton translocation through a membrane-bound, oligomycin-sensitive F0 subunit with ATP synthesis at the F1 subunit. The F0 subunit is typically composed of three proteins (F0−a, F0−b, and F0−c); however, the T. gondii genome has no obvious homologues for the F0−a and F0−b proteins (23). It was thus unclear whether the T. gondii F0 subunit is indeed associated with a putative membrane-bound F0 subunit or if this enzyme is localized in the mitochondrial matrix, as proposed previously for Plasmodium.
which is lacking all three parts of the F₀ subunit. The latter would imply that the proton motive force cannot be used for ATP synthesis. We showed by subcellular fractionation that the F₁ subunit is associated exclusively with the membrane fraction, which suggests an interaction of F₁ with a membrane-associated F₀ or F₀-like subunit. A conventional function of the T. gondii F₀F₁-ATPase in coupling the proton gradient with ATP synthesis is consistent with our observation that (i) an HDQ-mediated depolarization of the inner mitochondrial membrane leads to a ~30% reduction of the ATP level within
The close link between energy metabolism and stage conversion of the ATP level until these resources are reduced in the beginning of the inhibition process, for example, of glucose-6-phosphatase, might also contribute to a timely, limited stabilization of the ATP level until these resources are reduced in concentration.

The fraction of parasites with a detectable ΔΨm is not constant throughout the life cycle but is strongly decreased during tachyzoite-bradyzoite conversion. This is in agreement with the concept that T. gondii adapts its metabolism during the transition from tachyzoites to long-term persistent bradyzoites, which are believed to possess a reduced metabolism (4, 12, 41). The close link between energy metabolism and stage conversion is furthermore supported by our observation that long-term treatment with HDQ for 3 days leads to an upregulation of mRNA transcripts for bradyzoite markers. This is in agreement with previously reported observations that inhibitors of the respiratory chain and of oxidative phosphorylation lead to an induction of bradyzoite differentiation (4, 37). The parasite appears to respond in a situation of energy starvation with a differentiation into the dormant stage.

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REFERENCES

1. Baggish, A. L., and D. R. Hill. 2002. Antiparasitic agent atovaquone. Antimicrob. Agents Chemother. 46:163–173.

2. Biagini, G. A., P. O. Viriyavejakul, P. M. O’Neill, P. G. Bray, and S. A. Ward. 2006. Functional characterization and target validation of alternative complex I of Plasmodium falciparum mitochondria. Antimicrob. Agents Chemother. 50:1841–1851.

3. Bohne, W., J. Heesemann, and U. Gross. 1993. Induction of bradyzoite-specific Toxoplasma gondii antigens in gamma interferon-treated mouse macrophages. Infect. Immun. 61:1141–1145.

4. Bohne, W., J. Heesemann, and U. Gross. 1994. Reduced replication of Toxoplasma gondii is necessary for induction of bradyzoite-specific antigens: a possible role for nitric oxide in triggering stage conversion. Infect. Immun. 62:1761–1767.

5. Bohne, W., U. Gross, D. J. Ferguson, and J. Heesemann. 1995. Cloning and characterization of a bradyzoite-specifically expressed gene (hsp30/bag1) of Toxoplasma gondii, related to genes encoding small heat-shock proteins of plants. Mol. Microbiol. 16:1211–1280.

6. Brandt, U. 2006. Energy converting NADH:quinone oxidoreductases. Annu. Rev. Biochem. 75:69–92.

7. Crawford, M. J., N. Thomsen-Zieger, M. Ray, J. Schachter, D. S. Roos, and F. Seeger. 2006. Toxoplasma gondii scavenges host-derived lipidic acid despite its de novo synthesis in the apicoplast. EMBO J. 25:3214–3222.

8. Delocher, A., C. B. Hagen, J. E. Froehlich, J. E. Feagin, and M. Parsons. 2000. Analysis of targeting sequences demonstrates that trafficking to the Toxoplasma gondii plastidial branches off the secretory system. J. Cell Sci. 113:3969–3977.

9. Donald, R. G., and D. G. Roos. 1993. Stable molecular transformation of Toxoplasma gondii: a selectable dihydrofolate reductase-thymidylate synthase marker based on drug-resistance mutations in malaria. Proc. Natl. Acad. Sci. USA 90:11703–11707.

10. Dong, C. K., V. Patel, J. C. Yang, J. D. Dvorin, M. T. Durasingham, J. Clardy, and D. F. Wirth. 2009. Type II NADH dehydrogenase of the respiratory chain of Plasmodium falciparum and its inhibitors. Bioorg. Med. Chem. Lett. 19:972–975.

11. Dzierszinski, F., O. Popescu, C. Toursel, C. Sломянny, B. Yahiaoui, and S. Tomavo. 1999. The protozoan parasite Toxoplasma gondii expresses two functional plant-like glycolytic enzymes. Implications for evolutionary origin of apicomplexans. J. Biol. Chem. 274:24888–24895.

12. Dzierszinski, F., and L. J. Knoll. 2007. Biology of bradyzoites, p. 303–320. In J. M. Weiss and K. Kim (ed.), Toxoplasma gondii—the model apicomplexan: perspectives and methods. Academic Press, London, United Kingdom.

13. Eschemann, A., A. Galkin, W. Oettmeier, U. Brandt, and S. Kerscher. 2005. HDQ (1-hydroxy-2-dodecyl-4(1H)quinolone), a high affinity inhibitor for mitochondrial alternative NADH dehydrogenase. J. Biol. Chem. 280:3138–3142.

14. Fleige, T., K. Fischer, D. J. P. Ferguson, U. Gross, and W. Bohne. 2007. Carbohydrate metabolism in the Toxoplasma gondii apicoplast: localization of three glycolytic isoenzymes, the single pyruvate dehydrogenase complex, and a plastid phosphate translocator. Eukaryot. Cell 6:984–996.

15. Fleige, T., N. Pfaff, U. Groß, and W. Bohne. 2008. Localization of glucose-6-phosphate and TCA-cycle enzymes and first functional analysis of the TCA-cycle in Toxoplasma gondii. Int. J. Parasitol. 38:1121–1132.

16. Fisher, N., P. G. Bray, S. A. Ward, and G. A. Biagini. 2007. The malaria parasite type II NADH:quinone oxidoreductase: an alternative enzyme for an alternative lifestyle. Trends Parasitol. 23:305–310.

17. Fisher, N., P. G. Bray, S. A. Ward, and G. A. Biagini. 2008. Malaria-parasite mitochondrial dehydrogenase family as drug targets: too early to write the obituary. Trends Parasitol. 24:9–10.

18. Foth, B. J., L. M. Stimmmer, E. Handman, B. S. Crabb, A. N. Hodder, and G. I. McFadden. 2005. The malaria parasite Plasmodium falciparum has only one pyruvate dehydrogenase complex, which is located in the apicoplast. Mol. Microbiol. 55:39–53.

19. Fox, B. A., and D. J. Bzik. 2002. De novo pyrimidine biosynthesis is required for virulence of Toxoplasma gondii. Nature 415:926–929.

20. Jacobson, M. D., J. F. Burne, M. P. King, T. Miyashita, J. C. Reed, and M. C. Raff. 1993. Bcl-2 blocks apoptosis in cells lacking mitochondrial DNA. Nature 361:365–369.

21. Kerscher, S., V. Zickermann, and U. Brandt. 2008. The three families of mitochondrial NADH dehydrogenase family as drug targets: too early to write the obituary. Trends Parasitol. 24:9–10.

22. Kerscher, S., V. Zickermann, and U. Brandt. 2008. The three families of mitochondrial NADH dehydrogenase family as drug targets: too early to write the obituary. Trends Parasitol. 24:9–10.

23. Lin, S. S., S. Kerscher, A. Saleh, U. Brandt, U. Gross, and W. Bohne. 2008. The Toxoplasma gondii type-II NADH dehydrogenase TgNDH2-I is inhibited by 1-hydroxy-2-alkyl-4(1H)quinolones. Biochim. Biophys. Acta Bioenerg. 1777:1455–1462.

24. Mather, M. W., K. W. Henry, and A. B. Vaidya. 2002. Role of Apicoplasts in Plasmodium falciparum and its inhibitors. Bioorg. Med. Chem. Lett. 12:463–469.

25. Mather, M. W., A. B. Vaidya. 2007. Mitochondrial drug targets in apicomplexan parasites. Curr. Drug Targets 8:49–60.

26. Mather, M. W., and A. B. Vaidya. 2008. Mitochondria in malaria and related parasites: ancient, diverse and streamlined. J. Bioenerg. Biomembr. 40:125–133.

27. Meissner, M., S. Brehct, H. Bujard, and D. Soldati. 2001. Induction of myosin A expression by a newly established tetracycline repressor-based inducible system in Toxoplasma gondii. Nucleic Acid Res. 29:E115.

28. Meissner, M., D. Schluter, and D. Soldati. 2002. Role of Toxoplasma gondii myosin A in powering parasite gliding and host cell invasion. Science 298:837–840.

29. Melo, A. M., T. M. Bandeiras, and M. Teixeira. 2004. New insights into type II NAD(P)H:quinone oxidoreductases. Microbiol. Mol. Biol. Rev. 68:403–433.

30. Melo, E. J., M. Attias, and W. De Souza. 2000. The single mitochondrion of Toxoplasma gondii. J. Struct. Biol. 130:27–33.

31. Painter, H. J., J. M. Morrissey, M. W. Mather, and A. B. Vaidya. 2007.
Specific role of mitochondrial electron transport in blood-stage *Plasmodium falciparum*. Nature **446**:88–91.

30. Roos, D. S., R. G. Donald, N. S. Morrissette, and A. L. Moulton. 1994. Molecular tools for genetic dissection of the protozoan parasite *Toxoplasma gondii*. Methods Cell Biol. **45**:27–63.

31. Saleh, A., J. Friesen, S. Baumeister, U. Gross, and W. Bohne. 2007. Growth inhibition of *Toxoplasma gondii* and *Plasmodium falciparum* by nanomolar concentrations of 1-hydroxy-2-dodecyl-4(1H)quinolone, a high-affinity inhibitor of alternative (type II) NADH dehydrogenases. Antimicrob. Agents Chemother. **51**:1217–1222.

32. Seeber, F., D. J. P. Ferguson, and U. Gross. 1998. *Toxoplasma gondii*: a paraformaldehyde-insensitive diaphorase activity acts as a specific histochemical marker for the single mitochondrion. Exp. Parasitol. **98**:137–139.

33. Seeber, F., J. Limenitakis, and D. Soldati-Favre. 2008. Apicomplexan mitochondrial metabolism: a story of gains, losses and retentions. Trends Parasitol. **24**:468–478.

34. Soete, M., D. Camus, and J. F. Dubremetz. 1994. Experimental induction of bradyzoite-specific antigen expression and cyst formation by the RH strain of *Toxoplasma gondii* in vitro. Exp. Parasitol. **78**:361–370.

35. Soldati, D., and J. C. Boothroyd. 1993. Transient transfection and expression in the obligate intracellular parasite *Toxoplasma gondii*. Science **260**:349–352.

36. Striepen, B., M. J. Crawford, M. K. Shaw, L. G. Tilney, F. Seeber, and D. S. Roos. 2000. The plastid of *Toxoplasma gondii* is divided by association with the centrosomes. J. Cell Biol. **151**:1423–1434.

37. Tomavo, S., and J. C. Boothroyd. 1995. Interconnection between organellar functions, development and drug resistance in the protozoan parasite, *Toxoplasma gondii*. Int. J. Parasitol. **25**:1293–1299.

38. Vaidya, A. B., H. J. Painter, J. M. Morrissey, and M. W. Mather. 2008. The validity of mitochondrial dehydrogenases as antimalarial drug targets. Trends Parasitol. **24**:8–9.

39. Vercesi, A. E., C. O. Rodrigues, S. A. Uyemura, L. Zhong, and S. N. Moreno. 1998. Respiration and oxidative phosphorylation in the apicomplexan parasite *Toxoplasma gondii*. J. Biol. Chem. **273**:31040–31047.

40. Weinstein, E. A., T. Yano, L. S. Li, D. Avarbock, A. Avarbock, D. Helm, A. A. McColm, K. Duncan, J. T. Lonsdale, and H. Rubin. 2005. Inhibitors of type II NADH:menaquinone oxidoreductase represent a class of antitubercular drugs. Proc. Natl. Acad. Sci. USA **102**:4548–4553.

41. Weiss, L. M., and K. Kim. 2007. Bradyzoite development, p. 341–366. In L. M. Weiss and K. Kim (ed.), *Toxoplasma gondii*—the model apicomplexan: perspectives and methods. Academic Press, London, United Kingdom.