

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[∇]

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Received 20 July 2006/Returned for modification 25 October 2006/Accepted 3 January 2007

Both apicomplexan parasites *Toxoplasma gondii* and *Plasmodium falciparum* lack type I NADH dehydrogenases (complex I) but instead carry alternative (type II) NADH dehydrogenases, which are absent in mammalian cells and are thus considered promising antimicrobial drug targets. The quinolone-like compound 1-hydroxy-2-dodecyl-4(1H)quinolone (HDQ) was recently described as a high-affinity inhibitor of fungal alternative NADH dehydrogenases in enzymatic assays, probably by interfering with the ubiquinol binding site of the enzyme. We describe here that HDQ effectively inhibits the replication rates of *P. falciparum* and *T. gondii* in tissue culture. The 50% inhibitory concentration (IC₅₀) of HDQ for *T. gondii* was determined to be 2.4 ± 0.3 nM with a growth assay based on vacuole sizes and 3.7 ± 1.4 nM with a growth assay based on beta-galactosidase activity. Quantification of the *P. falciparum* replication rate using a fluorometric assay revealed an IC₅₀ of 14.0 ± 1.9 nM. An important feature of the HDQ structure is the length of the alkyl side chain at position 2. Derivatives with alkyl side chains of C₆, C₈, C₁₂ (HDQ), and C₁₄ all displayed excellent anti-*T. gondii* activity, while a C₅ derivative completely failed to inhibit parasite replication. A combined treatment of *T. gondii*-infected cells with HDQ and the antimalarial agent atovaquone, which blocks the ubiquinol oxidation site of cytochrome *b* in complex III, resulted in synergism, with a calculated fractional inhibitory concentration of 0.16 nM. Interference of the mitochondrial ubiquinone/ubiquinol cycle at two different locations thus appears to be a highly effective strategy for inhibiting parasite replication. HDQ and its derivatives, particularly in combination with atovaquone, represent promising compounds with a high potential for antimalarial and antitoxoplasmal therapy.

Diseases caused by apicomplexan parasites, such as *Plasmodium* and *Toxoplasma* species, are among the most serious health problems in the world, leading to an estimated 1 to 3 million deaths per year just due to malaria. The emerging resistance against established drugs in *Plasmodium* populations emphasizes the need to continuously identify and evaluate novel targets for drug therapy (9, 14).

A pathway which is well known as an effective drug target against malaria and toxoplasmosis is the mitochondrial electron transport chain. Several components of the electron transport chain in *Plasmodium* and *Toxoplasma* display significant differences from their mammalian counterparts which can be exploited for chemotherapy (4, 22–24). Atovaquone is a recently developed antimalarial agent which inhibits electron transport at the bc₁ complex (complex III) by interfering with the ubiquinol oxidation site of cytochrome *b* (19, 20, 25). The resulting collapse of the mitochondrial membrane potential (19) is lethal for the parasite.

The most fundamental difference in the architectures of the human and *Plasmodium* electron transport chains is in the activity of NADH:quinone oxidoreductase, which is also called

NADH dehydrogenase (2, 8). Mammals possess a rotenone-sensitive type I NADH dehydrogenase (multisubunit complex I), which is localized inside the inner mitochondrial membrane. In contrast, genomic data reveal that *Plasmodium* species and *Toxoplasma gondii* lack complex I but instead possess an alternative (type II) NADH dehydrogenase(s), which is encoded by a single gene (P_fndh2 [GenBank accession no. PFI0735c]) in *Plasmodium falciparum* and by two genes in *T. gondii* (T_gndh2-I [accession no. DQ211932] and T_gndh2-II [accession no. DQ228957]) (8; A. Saleh, unpublished data; <http://www.toxodb.org>).

Alternative NADH dehydrogenases are rotenone-insensitive single-subunit enzymes and are present in the plasma membranes of many bacteria and in the inner mitochondrial membranes of various fungi and plants and some protozoa (10, 13, 16). The rotenone insensitivity of NADH:quinone oxidoreductase activity in *Plasmodium* species and *T. gondii* was confirmed in biochemical assays (2, 22, 25). In contrast to the case for type I enzymes, the transfer of electrons from NADH to quinones by alternative NADH dehydrogenases is not coupled to proton translocation. The absence of alternative NADH dehydrogenases in mammalian cells defines these enzymes as promising antimicrobial drug targets. A type II NADH:menaquinone oxidoreductase inhibitor was shown to interfere with *Mycobacterium tuberculosis* growth (26, 27). For *P. falciparum*, micromolar concentrations of low-affinity flavin

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[∇] Published ahead of print on 22 January 2007.

reagents were shown to inhibit PfNDH2 activity, to collapse the parasite's mitochondrial membrane potential, and to inhibit *P. falciparum* replication (2).

Recently, the compound 1-hydroxy-2-dodecyl-4(1*H*)quinolone (HDQ) was identified in enzymatic assays as a high-affinity inhibitor of the alternative NADH dehydrogenase from the fungus *Yarrowia lipolytica* (5). In this study, we describe that HDQ is a highly effective drug for inhibiting the replication of *P. falciparum* and *T. gondii* in nanomolar concentrations and that it acts in synergism with atovaquone.

MATERIALS AND METHODS

***T. gondii* strains and cultivation.** Parasites were propagated in human foreskin fibroblasts (HFF) as previously described (17). A clonal isolate of the RH strain was used for quantification of growth rates by immunofluorescence microscopy. A *lacZ*-transfected RH strain (3) was used for beta-galactosidase-based quantification of replication rates.

Cultivation of *Plasmodium falciparum*. The *P. falciparum* isolate FCBR (15) was cultivated in RPMI 1640 medium supplemented with 10% human plasma and human erythrocytes of blood group A (rhesus factor positive), as described elsewhere (21). For maintenance of the culture and for drug susceptibility assays, parasites were cultured in T25 flasks in a total volume of 10 ml. Flasks were flushed with gas composed of 90% nitrogen, 5% O₂, and 5% CO₂. Cultures were synchronized by the sorbitol method, as described elsewhere (11).

Drugs. HDQ (Fig. 1) and all other 1-hydroxy-2-alkyl-4(1*H*)quinolone derivatives were kindly provided by Walter Oettmeier (Ruhr-Universität, Bochum, Germany). The derivatives were dissolved in tissue culture-grade dimethyl sulfoxide (DMSO) at a concentration of 2.5 mM. All further dilutions were performed in tissue culture medium. Atovaquone was dissolved in DMSO at a concentration of 5 mM. Non-drug-treated controls of *T. gondii*- and *P. falciparum*-infected cultures were incubated with DMSO at identical concentrations to those used for drug-treated samples. Due to the limited amounts of C₅, C₆, C₈, and C₁₄ derivatives, most of the experiments were performed with HDQ.

Replication rate determined by immunofluorescence microscopy. HFF monolayers were infected for 2 h with freshly lysed *T. gondii* parasites, washed to remove extracellular parasites, and subsequently treated with drugs or a DMSO control. After 24 h of drug treatment, samples were fixed with 4% paraformaldehyde-phosphate-buffered saline (PBS) for 10 min and permeabilized with 0.25% Triton X-100-PBS for 15 min. After being blocked for 1 h with PBS-1% bovine serum albumin (BSA), samples were incubated for 1 h with a polyclonal rabbit anti-*T. gondii* antiserum diluted 1:2,000 in PBS-1% BSA, followed by incubation for 1 h with a Cy3-conjugated anti-rabbit immunoglobulin G (1:250 in PBS-1% BSA; Dianova). The parasite number per vacuole was determined from at least 100 parasitophorous vacuoles by immunofluorescence microscopy.

Replication rate determined by beta-galactosidase activity. The beta-galactosidase activity of *T. gondii* parasites which are stably transfected with the *Escherichia coli lacZ* gene can be used to accurately quantify the parasite replication rate by using a colorimetric assay (12). Freshly lysed parasites of a beta-galactosidase-expressing RH strain (3) were used to infect HFF monolayers (1 × 10⁴ parasites/well) grown in 24-well plates in 1% Dulbecco's modified Eagle's medium without phenol red for 4 h at 37°C. The infected monolayers were washed twice with 1% Dulbecco's modified Eagle's medium without phenol red and incubated with different concentrations of HDQ in duplicate (1, 0.1, 0.01, and 0.001 μM) and with a final concentration of 100 μM chlorophenol red-β-D-galactopyranoside at 37°C. Absorbance at 570 nm and 630 nm was determined photometrically.

Quantification of *P. falciparum* replication rate by using a fluorometric assay. An 800-μl aliquot of a *P. falciparum* culture was added to 1 ml 0.08% saponin in PBS in order to lyse red blood cells. Subsequent sample preparation steps were performed as previously described (18). Briefly, samples were centrifuged at 15,800 × g for 4 min, and the supernatants were carefully removed. The parasite pellets were solubilized in 25 μl of a solution (pH 5.5) containing 6 M guanidium-HCl and 6 M sodium acetate. Afterwards, 2 ml of a solution containing 2 M NaCl, 50 mM Tris-HCl (pH 7.8), and 0.33 μg/ml of Hoechst 33258 was added. Finally, 50 μl of a 24:1 mixture of chloroform-isoamyl alcohol was added, and samples were mixed by vortexing. After centrifugation at 15,800 × g for 1 min, 1.9 ml of the supernatant was transferred to a 24-well plate. Triplicates of each sample were measured in a fluorometer (Victor 5; Wallace) at wavelengths of 340 and 460 nm. Each well was measured at two different spots.

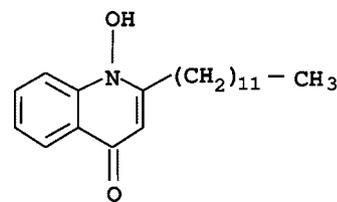


FIG. 1. Structure of HDQ.

Host cell metabolic assay. The metabolic activity of the host cells was tested by using an AlamarBlue assay (Biosources International Inc., Camarillo, CA). AlamarBlue is an indicator dye formulated to quantitatively measure the proliferation of a variety of human or animal cells, bacteria, mycobacteria, or fungi. It consists of an oxidation-reduction (redox) indicator that yields a colorimetric change and a fluorescent signal in response to metabolic activity. Briefly, HFF were grown to confluence in 24-well plates, treated for 48 h with different concentrations of HDQ, and processed according to the instructions provided by the manufacturer.

Genome data mining. Preliminary genomic and/or cDNA sequence data were accessed via <http://toxodb.org> (version 3.0) and/or http://www.tigr.org/tdb/t_gondii/.

RESULTS

HDQ inhibits *T. gondii* replication. The quinolone compound HDQ (Fig. 1) was recently shown to inhibit the activity of the mitochondrial alternative (type II) NADH dehydrogenase of the fungus *Yarrowia lipolytica* in enzymatic assays (5). We tested the potential of this drug to inhibit the replication of the apicomplexan parasites *T. gondii* and *P. falciparum*, which both lack the type I NADH oxidoreductase (complex I) but instead carry alternative NADH dehydrogenases. A confluent monolayer of HFF was infected with tachyzoites of the *T. gondii* RH strain and treated with various concentrations of HDQ ranging from 1 nM to 10 μM. Two growth assays were applied to quantify the *T. gondii* replication rate. First, the average number of parasites per vacuole was determined at 24 h postinfection by immunofluorescence microscopy. In untreated cells, parasites completed up to four replication cycles, with 58% of vacuoles containing 8 parasites and 22% containing 16 parasites. In contrast, nanomolar concentrations of HDQ were found to effectively inhibit *T. gondii* replication, with a 50% inhibitory concentration (IC₅₀) of 2.4 ± 0.3 nM (Fig. 2A). Ninety percent of the parasites in samples treated with >10 nM HDQ were located in vacuoles containing only a single parasite, suggesting that the drug acts immediately on the parasite. Moreover, immunofluorescence analysis of the intracellular *T. gondii* parasites treated with 10 μM HDQ showed the presence of abnormal parasites which lost their typical crescent shape (data not shown). Invasion was not inhibited by HDQ, since pretreatment of extracellular parasites with 1 to 1,000 nM HDQ for 2 h before infection did not result in a decreased infection rate (data not shown). For the second independent growth assay, a beta-galactosidase-expressing *T. gondii* RH strain (3) was used to infect an HFF monolayer, and the replication rate was determined at 30 h postinfection by using a colorimetric assay (Fig. 2B). The replication rate of *T. gondii* was dose-dependently inhibited by HDQ, with an IC₅₀ of 3.7 ± 1.4 nM, which is in good agreement with the results from the microscopic growth assay.

To analyze the potential effects of HDQ on the host cell, we

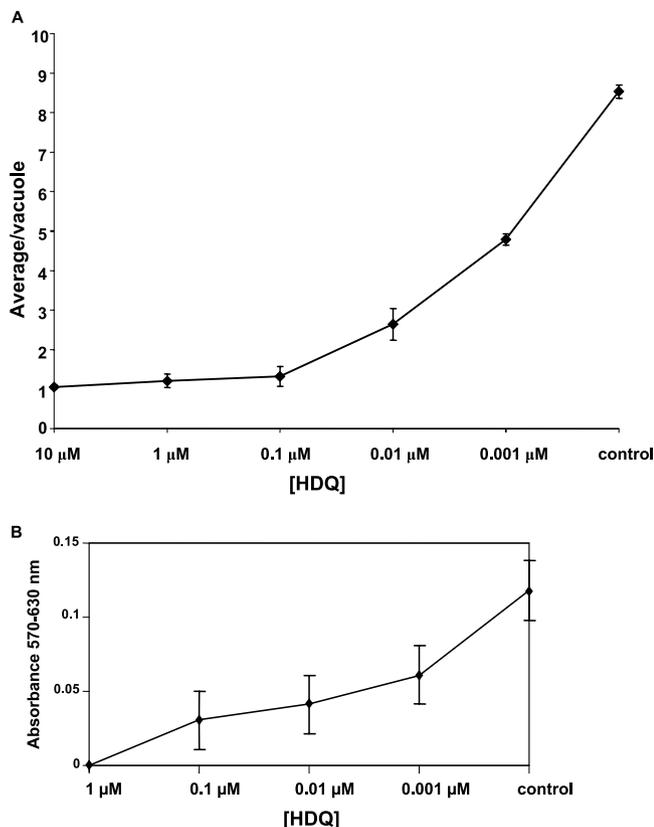


FIG. 2. HDQ inhibits the *T. gondii* replication rate. (A) HFF monolayers were infected with *T. gondii* and treated with either the indicated concentrations of HDQ or a DMSO control. After 24 h of drug treatment, the average number of parasites per vacuole was determined by immunofluorescence microscopy for two independent experiments and given as the mean \pm standard deviation (SD). (B) HFF monolayers were infected with a beta-galactosidase-expressing *T. gondii* RH strain (3) and treated with either the indicated concentrations of HDQ or a DMSO control. Beta-galactosidase activity was determined at 30 h postinfection, using a colorimetric assay. The figure shows the means \pm SD of optical densities at 570 to 630 nm for duplicates.

tested the metabolic activity of host cells with the AlamarBlue assay. No difference between HDQ-treated and untreated cells was found, indicating that HDQ at the applied concentrations of up to 10 μ M has no inhibitory effect on host cell metabolism.

HDQ inhibits *P. falciparum* replication. To determine the HDQ susceptibility of *P. falciparum*, ring-stage parasites were cultivated in the presence of HDQ at concentrations of 1 μ M, 100 nM, 10 nM, and 1 nM for 48 h. Like the case for *Toxoplasma gondii*, HDQ treatment resulted in a dose-dependent decrease of parasitemia, as determined with Giemsa-stained blood smears (Fig. 3A). A reduction of the parasitemia to 50% of the control level was achieved at an HDQ concentration of 54 ± 25 nM. To accurately determine the IC_{50} of HDQ for *P. falciparum*, a quantitative growth assay was applied, based on binding of the fluorescent Hoechst 33258 dye to parasite DNA (18). Cultures of young trophozoites with an initial parasitemia of 2% were incubated for 36 h in the presence of 1 μ M, 100 nM, 10 nM, or 1 nM HDQ. Chloroquine-diphosphate at 2 μ M, a concentration which completely inhibits parasite replication,

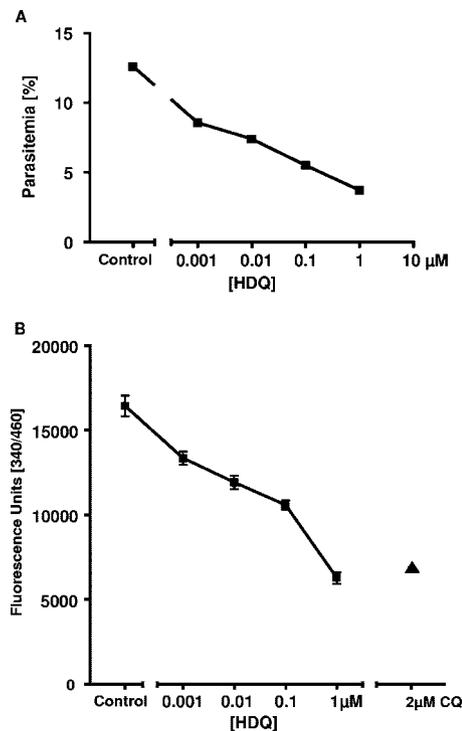


FIG. 3. (A) HDQ treatment of *P. falciparum* cultures results in reduced parasitemia. Sorbitol-synchronized ring-stage parasites of the *P. falciparum* FCBR isolate (initial parasitemia, 3.2%) were incubated with the indicated concentrations of HDQ in T25 flasks. DMSO was added to untreated controls to identical concentrations as those in drug-treated samples. The final parasitemia was determined 48 h later for Giemsa-stained blood smears by counting 1,000 to 2,000 red blood cells. The values represent the means of duplicates from a representative experiment. HDQ dose-dependently inhibits *P. falciparum* growth, with a 50% reduction of parasitemia at 54.7 ± 25.2 nM. (B) IC_{50} determinations for HDQ with *Plasmodium falciparum*. Young trophozoites (2% parasitemia) were incubated for 36 h in T25 flasks in the presence of the indicated HDQ concentrations. Each HDQ concentration was tested in triplicate. DMSO was added to untreated controls to the same concentration as that used for drug-treated samples. Chloroquine-diphosphate (CQ; 2 μ M) was used in one sample to completely block parasite replication. Sample preparation for the fluorometric assay was performed as previously described (18). Samples were measured in a fluorometer (Victor 5; Wallac) at wavelengths of 340 and 460 nm. The means of triplicates \pm SD are given as fluorescence units. The IC_{50} of HDQ for *P. falciparum* is 14.0 ± 1.9 nM and was defined as the HDQ concentration which results in a fluorescence signal which is exactly between the signal of the untreated control and that of the chloroquine-diphosphate-treated sample.

was used as a control to determine the background fluorescence of nonreplicating parasites. The fluorescence intensity decreased dose dependently with increasing HDQ concentrations, indicating that parasite replication was effectively inhibited. The IC_{50} of HDQ for *P. falciparum* was determined to be 14.0 ± 1.9 nM (Fig. 3B).

HDQ acts in synergism with atovaquone. Atovaquone is a well-established drug for treating toxoplasmosis and malaria. We investigated a potential synergistic effect of HDQ with atovaquone on the inhibition of *T. gondii* replication. The combination of both drugs resulted in a stronger inhibition of parasite replication than treatments with the individual drugs (Fig. 4). Synergism was evaluated as the sum of the fractional

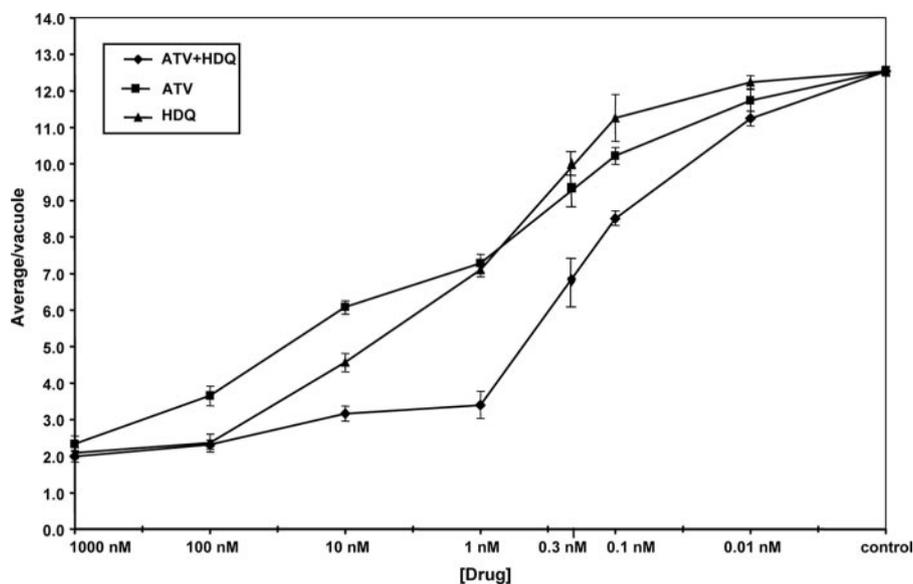


FIG. 4. HDQ acts in synergism with atovaquone. An HFF monolayer was infected with tachyzoites of the *T. gondii* RH strain and treated with the indicated concentrations of HDQ and atovaquone (ATV), either alone or in combination. The average number of parasites per parasitophorous vacuole was determined at 36 h postinfection for at least 100 vacuoles for triplicate samples by immunofluorescence microscopy and is given as the mean \pm SD.

inhibitory concentrations (sum FIC) by the following equation: sum FIC = (IC₅₀ of drug A in combination/IC₅₀ of drug A alone) + (IC₅₀ of drug B in combination/IC₅₀ of drug B alone). The IC₅₀ values determined from Fig. 4 were 9 nM for atovaquone, 4 nM for HDQ, and 0.45 nM for both in combination. The calculation of the sum FIC of atovaquone and HDQ based on the above formula is as follows: (0.45/9) + (0.45/4) = 0.16. A sum FIC of <0.5 represents synergism, one of >1 represents antagonism, and one equal to 1 represents addition (1). Therefore, the calculated sum FIC of 0.16 indicates synergism between atovaquone and HDQ.

The length of the alkyl side chain at position 2 is important for drug action. Beside HDQ, which possesses a long hydrophobic (CH₂)₁₁-CH₃ (C₁₂) side chain at position 2, further 1-hydroxy-2-alkyl-4(1)quinolone derivatives with different alkyl side chain lengths were tested for the ability to inhibit *T. gondii* replication. Derivatives with alkyl side chains of (CH₂)₅-CH₃ (C₆), (CH₂)₇-CH₃ (C₈), and (CH₂)₁₃-CH₃ (C₁₄) inhibited the growth of *T. gondii* as effectively as HDQ (C₁₂) at concentrations of 0.1 μ M and 0.01 μ M (Fig. 5). In contrast, a derivative with the alkyl side chain (CH₂)₄-CH₃ (C₅) did not inhibit *T. gondii* replication. This suggests that the alkyl side chain at position 2 is essential for the inhibitory action of 1-hydroxy-2-alkyl-4(1)quinolone derivatives and needs to have a minimal side chain of -(CH₂)₅-CH₃ (C₆).

Recovery of parasite replication after 24 h of HDQ treatment. To further characterize the antiparasitic activity of the drug, we incubated *T. gondii*-infected HFF for 24 h with either 0.1 or 1 μ M HDQ, followed by cultivation for another 72 h without the drug. The ability of parasites to recover from the drug treatment was then determined by using the number of parasites per parasitophorous vacuole as a parameter for viability. Continuous treatment for 96 h with 0.1 and 1 μ M HDQ resulted in an almost complete inhibition of parasite replica-

tion in which most parasitophorous vacuoles harbored one or two parasites, very rarely a maximum of four parasites was observed, and none of the vacuoles contained eight or more parasites. For the recovery experiment, a vacuole with eight parasites was thus considered to contain replicating parasites (Fig. 6). In samples treated with 0.1 μ M HDQ for 24 h, almost 50% of the vacuoles recovered from HDQ treatment. This number increased to 65.8% when the 24-h HDQ treatment started with a delay of 6 h postinfection. However, when the HDQ concentration was increased to 1 μ M, the recovery strongly decreased, to <3%, suggesting that at this concentra-

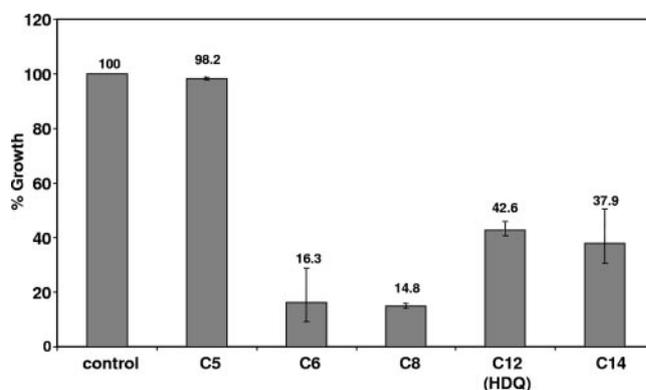


FIG. 5. Susceptibility of *T. gondii* to various 1-hydroxy-2-alkyl-4(1)quinolone derivatives. HFF monolayers were infected with a beta-galactosidase-expressing *T. gondii* RH strain (3) and treated with 10 nM 1-hydroxy-2-alkyl-4(1)quinolone derivatives with alkyl side chain lengths of C₅, C₆, C₈, C₁₂ (HDQ), and C₁₄. Beta-galactosidase activity was determined at 30 h postinfection, using a colorimetric assay. Bars in the figure show the growth of drug-treated samples as percentages of the growth of untreated controls. Means from two independent experiments \pm SD are given.

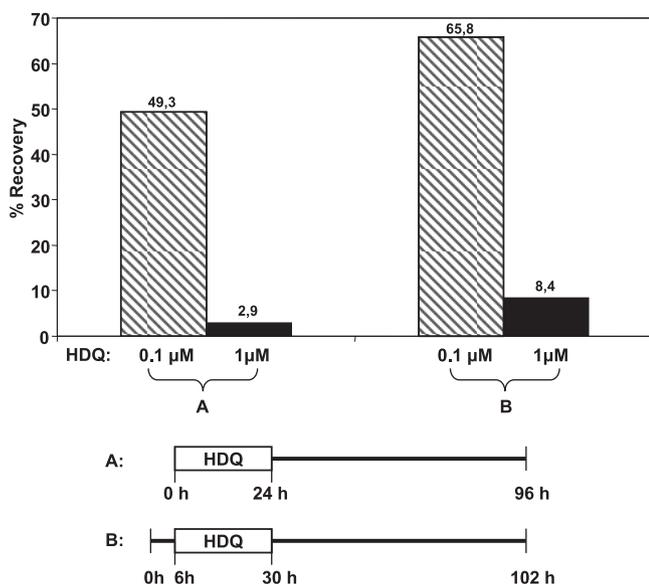


FIG. 6. Recovery of parasite growth after 24 h of HDQ treatment. *T. gondii* (strain RH)-infected HFF were treated for the indicated times (A and B) with either 0.1 or 1 μM HDQ, followed by cultivation for another 72 h without the drug. The sizes of the parasitophorous vacuoles in the samples were determined by immunofluorescence microscopy. Parasitophorous vacuoles with eight or more parasites were considered to contain viable parasites that recovered from drug inhibition. The figure indicates the fractions of vacuoles containing eight or more parasites of the total number of vacuoles from a representative experiment. In samples treated with 0.1 μM HDQ, about 50% of the vacuoles recovered from HDQ treatment. In contrast, the recovery rate strongly decreased, to <3%, after treatment with 1 μM HDQ.

tion a 24-h HDQ treatment exerts a predominantly parasitocidal activity.

DISCUSSION

Due to their absence in mammalian cells, alternative NADH dehydrogenases are recognized as important antimicrobial drug targets (2, 26). The compound HDQ was recently described as the first high-affinity inhibitor of alternative NADH dehydrogenases, with an IC_{50} in enzymatic assays of 200 nM (5). We report in this study that HDQ is able to inhibit replication of the apicomplexan parasites *P. falciparum* and *T. gondii* in the nanomolar range. Both parasites lack a conventional complex I in their electron transport chains and instead possess alternative NADH dehydrogenases.

Although it cannot be ruled out completely that HDQ has additional effects on *T. gondii* and *P. falciparum* which are unrelated to alternative NADH dehydrogenase inhibition, the observed synergism between HDQ and the complex III inhibitor atovaquone suggests that HDQ, like atovaquone, affects the mitochondrial electron transport chain. This synergism is most likely due to the inhibition of the ubiquinone/ubiquinol cycle at two different locations, i.e., the reduction site (alternative NADH dehydrogenase) for HDQ and the oxidation site (complex III) for atovaquone. A synergism of atovaquone with low-affinity inhibitors of alternative NADH dehydrogenases was recently shown for *Plasmodium falciparum*, demonstrating that simultaneous inhibition of the ubiquinone/ubiquinol cycle

at different points has a huge pharmacological potential (2). However, the general flavines used in the previous study, such as diphenylene iodonium chloride, have an IC_{50} which is 500-fold higher than that of HDQ and are most likely unsuitable for clinical studies (2, 5).

In contrast, the IC_{50} of HDQ in the low nanomolar range makes HDQ a highly attractive candidate for further studies, particularly since we did not observe any negative effects on the human host cells at the applied concentrations. HDQ and alkyl side chain derivatives thus represent promising compounds with a high potential for antimalarial and antitoxoplasmal therapy. In addition to *Toxoplasma* and *Plasmodium*, HDQ might be effective against a broader spectrum of pathogens/parasites, including other apicomplexan parasites with a mitochondrial respiratory chain as well as some kinetoplastids, such as *Trypanosoma*, which express type II NADH dehydrogenases in addition to a conventional complex I (6, 7).

It has to be mentioned that HDQ in the nanomolar range is not able to kill *T. gondii* parasites but rather has a static effect on the parasites. Intracellular parasites which were treated with 10 nM HDQ for 48 h were almost completely able to recover from HDQ-mediated growth arrest and to continue replication after drug removal. If the parasites were treated with 10 μM HDQ, however, a parasitocidal effect was observed. At this high concentration, which is >2,000-fold higher than the IC_{50} , side effects of HDQ on targets which are unrelated to the alternative NADH dehydrogenases cannot be excluded.

A critical feature of the 1-hydroxy-2-alkyl-4(1)quinolone structure is the length of the alkyl side chain at position 2. While derivatives with alkyl side chains of C_6 , C_8 , C_{12} (HDQ), and C_{14} all displayed excellent antiparasitic activities in the nanomolar range, a C_5 derivative completely failed to inhibit *T. gondii* replication. A minimal alkyl side chain length of C_6 thus appears to be required for drug action. Due to their structural similarities, it is most likely that 1-hydroxy-2-alkyl-4(1)quinolones compete with ubiquinones for the same binding site in alternative NADH dehydrogenases (5). However, it has to be mentioned that steady-state inhibition kinetics of HDQ on the *Y. lipolytica* alternative NADH dehydrogenase displayed a noncompetitive pattern for the hydrophobic ubiquinone derivative *n*-decylubiquinone, which the authors of the study used as an electron acceptor (5). This unexpected finding was explained by a proposed ping-pong mechanism for the two-substrate reaction of the enzyme (5). Future biochemical analysis will reveal whether HDQ exhibits the same inhibition mode on the *T. gondii* and *P. falciparum* orthologs as on the *Y. lipolytica* enzyme.

ACKNOWLEDGMENTS

We thank Walter Oettmeier, University of Bochum, for providing us with 1-hydroxy-2-alkyl-4(1)quinolone derivatives and Klaus Lingelbach, University of Marburg, for support with *P. falciparum* tissue cultures.

Ahmad Saleh is supported by a DAAD (Deutscher Akademischer Austauschdienst) scholarship. This work was supported by a grant from the Deutsche Forschungsgemeinschaft (BO 1557/3-1). Genomic data were provided by The Institute for Genomic Research (supported by NIH grant AI05093) and by the Sanger Center (Wellcome Trust). Expressed sequence tag sequences were generated by Washington University (NIH grant 1R01AI045806-01A1).

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