

The Effects of Antimalarials on the *Plasmodium falciparum* Dihydroorotate Dehydrogenase

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ITTARAT, I., ASAWAMAHASAKDA, W., AND MESHNICK, S. R. 1994. The effects of anti-malarials on the *Plasmodium falciparum* dihydroorotate dehydrogenase. *Experimental Parasitology* 79, 50-56. Dihydroorotate dehydrogenase (DHOD) is a key enzyme in *de novo* pyrimidine biosynthesis and the major source of electrons for the mitochondrial electron transport chain of intraerythrocytic malaria parasites. DHOD and the electron transport chain may also be the site of inhibition by certain antimalarial drugs. In order to test this, *Plasmodium falciparum*-infected erythrocytes were exposed *in vitro* to artemisinin or various 8-aminoquinolines, such as primaquine, WR 238605, WR 225448, and WR 255956, and then assayed for both enzyme activity and [³H]hypoxanthine incorporation, which is an indicator of viability. Atovaquone inhibits DHOD activity to a much greater extent than hypoxanthine incorporation, which is consistent with previous reports that it targets the parasite respiratory chain. However, artemisinin and the 8-aminoquinolines inhibit DHOD to the same or lesser extent than hypoxanthine incorporation, suggesting that these compounds have different modes of action. © 1994 Academic Press, Inc.

INDEX DESCRIPTORS AND ABBREVIATIONS: *Plasmodium falciparum*; Protozoa; Malaria; Antimalarial; Primaquine; Artemisinin; Dihydroorotate dehydrogenase (L-4,5-dihydroorotate:oxygen oxidoreductase, EC 1.3.3.1; DHOD).

INTRODUCTION

DHOD catalyzes the conversion of dihydroorotate to orotate (Krooth *et al.* 1969; Miller and Kerr 1967), which is an important reaction in the pyrimidine biosynthetic pathway. This pathway is a potential target for antimalarial drugs (Gero *et al.* 1984; Prapunwattana *et al.* 1988; Rathod *et al.* 1989) since malaria parasites lack the alternative pyrimidine salvage pathway (Walsh and Sherman 1968). DHOD has been shown to be coupled to the malarial mitochondrial electron transport system in *P. knowlesi*, *P. berghei*, and *P. gallinaceum* (Chen and Jones 1976; Gutteridge *et al.* 1979; Gero and O'Sullivan 1985; Hines *et al.* 1986). DHOD has been purified and characterized in *P. berghei* (Krungkrai *et al.* 1991) and *P. falciparum* (Krungkrai *et al.* 1992). Recently, the DHOD gene homologue of *P. falciparum* has been cloned and sequenced (Le Blanc and Wilson, 1993).

Several antimalarial drugs may target the parasite mitochondria, including artemisinin derivatives (Ellis *et al.* 1985; Jiang *et al.* 1985; Maeno *et al.* 1993), atovaquone (Fry and Pudney 1992), and various 8-aminoquinolines (Howells *et al.* 1970; Aikawa and Beaudoin 1970; Peters *et al.* 1984; Warhurst 1984) (Fig. 1). We report here the action of these drugs (Fig. 1) on the *P. falciparum* DHOD.

MATERIALS AND METHODS

Drugs. WR 238605, WR 225448, and WR 255956 were obtained from the Division of Experimental Therapeutics, Walter Reed Army Institute of Research (Washington, DC). Atovaquone (BW 566c80) was a gift from the Burroughs-Wellcome Co. (Research Triangle Park, NC). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. The structures of all drugs used are shown in Fig. 1.

Drug treatment of parasites. *P. falciparum*, strain FCR-3, was cultivated in candle jars by the method of

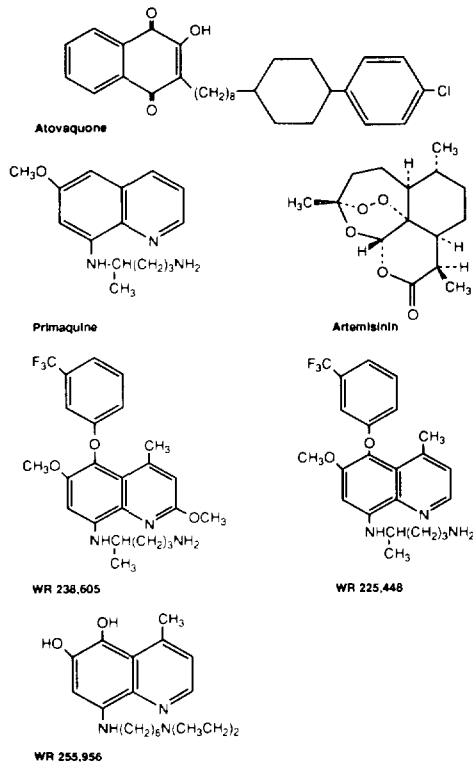


FIG. 1. Chemical structures of the antimalarial drugs tested.

Trager and Jensen (1976) in RPMI 1640 (Gibco, Grand Island, NY) containing 10% human serum (v/v), 25 mM HEPES, and 32 mM sodium bicarbonate. Parasites were synchronized by sorbitol lysis (Lambros and Vanderberg 1979).

Malaria-infected red cells were incubated at 37°C with various concentrations of antimalarial drugs in culture plates containing 20 ml of medium and 7% hematocrit. Each plate had a 20–30% parasitemia containing 75–85% trophozoites. Immediately after adding the drug, a 100- μ l aliquot of each culture was transferred to a well in a microtiter plate to which 1 μ Ci of [3 H]hypoxanthine was added. The microtiter plate was harvested after 6 hr at 37°C using a Brandel cell harvester Model M24 (Brandel, Gaithersburg, MD) and counted on a Beckman LS 7000 scintillation counter (Beckman Instruments, Fullerton, CA) using Scintiverse BD (Fisher Scientific, Fair Lawn, NJ) as scintillant.

After the 100- μ l aliquot was removed, the culture plates were incubated for 6 hr at 37°C. The cell suspension was then removed and centrifuged at 2000g for 5 min, and the resulting cell pellet was washed 3 times with Dulbecco's phosphate-buffered saline. Isolated parasites were then obtained from the pellet by

saponin lysis (Fairfield *et al.* 1983) and washed 4 times with Dulbecco's phosphate-buffered saline. DHOD was then assayed in isolated parasites as described below.

Analysis of plasmodial DHOD. DHOD was assayed by a modification of a high-performance liquid chromatography (HPLC) method described previously (Ittarat *et al.* 1992). The parasite pellet isolated from each plate was suspended in 20 μ l of 10 mM HEPES-KOH buffer, pH 8.0, and then immediately mixed with 180 μ l of the prewarmed reaction mixture comprising the same buffer plus dihydroorotate (250 μ M) and tryptophan (30 μ M) as an internal standard. This mixture was incubated for 25 min at 37°C and then stopped by boiling as previously described (Ittarat *et al.* 1992). The rate of formation of orotate was measured by using a Rainin Rabbit HPLC (Rainin Instrument Co., Woburn, MA) connected to a Macintosh computer loaded with Dynamax HPLC Method Manager Version 1.3 software. The column used was prepacked Hypersil 5 C₁₈ with a guard column (Phenomenex, Torrance, CA). The mobile phase consisted of 3 mM PIC A (Waters Assoc., Milford, MA) in 5 mM ammonium dihydrogenphosphate, pH 6.0, containing 5% methanol. The column was eluted isocratically at a flow rate of 1 ml/min and monitored by uv absorption at 260 nm. A standard curve was constructed for the ratios of the areas of the orotate and tryptophan peaks.

The validity of the assay was tested by determining DHOD activities of parasites obtained from cultures containing various parasitemias. Protein was determined using the D_C protein assay (BioRad, Rockville Center, NY) with bovine serum albumin as a standard. Unpaired one-tailed *t* tests were performed using Microsoft Excel Version 4.0.

RESULTS

Enzymatic activity in *P. falciparum*. DHOD could be assayed reproducibly by HPLC. The product, orotate, and the internal standard, tryptophan, elute from the column at 16.3 ± 0.5 min and 18.5 ± 0.5 min, respectively (Fig. 2). Under the HPLC conditions used, no peak was seen for dihydroorotate. DHOD activity was measured in parasite suspensions obtained from cultures with various parasitemias. A linear relationship between enzyme activity and parasite protein ($R^2 = 0.998$) was found (data not shown).

Effects of antimalarials on parasites. DHOD activity and [3 H]hypoxanthine uptake were measured in cultures which were

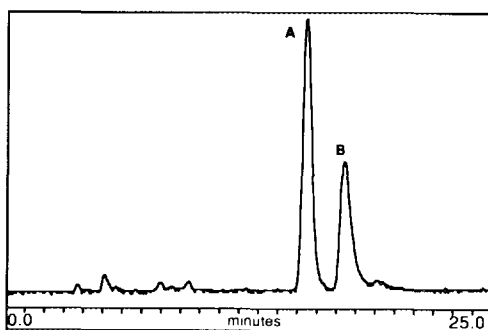


FIG. 2. Chromatogram of assay mixture. (A) Orotate; (B) tryptophan.

treated with various concentrations of anti-malarial drugs (Fig. 3).

Very low concentrations of atovaquone (5 nM) inhibit 90% of DHOD activity while inhibiting hypoxanthine uptake by less than 30%. Thus, atovaquone affects parasite enzyme activity at concentrations which had little effect on parasite viability.

Artemisinin, on the other hand, inhibits DHOD activity and hypoxanthine uptake to similar extents at concentrations between 20 and 500 nM. The degree of inhibition ranges from 4 to 49%.

The 8-aminoquinolines inhibit hypoxanthine uptake better than DHOD. At 50 μ M concentrations, primaquine, WR 238605, WR 225448, and WR 255956 inhibit parasite hypoxanthine uptake by 59–80% but inhibit DHOD by only 23–39%. For each drug, the difference is statistically significant, with *P* values of 0.0006, 0.0007, 0.0004, and 0.002, respectively. At 10 μ M, primaquine, WR 238605, and WR 255956 hardly have any effect on DHOD, but inhibit hypoxanthine uptake by 28 to 35%. For each of these drugs, the differences are also statistically significant, with *P* values of 0.009, 0.0001, and 0.0002, respectively. WR225448 has a similar effect at 2.5 μ M, inhibiting hypoxanthine uptake by 30% without affecting DHOD (*P* < 0.00001).

DISCUSSION

In the present study we found that atovaquone, a hydroxynaphthoquinone, inhibits

DHOD at concentrations which barely inhibit [³H]hypoxanthine uptake. This is consistent with previous reports, which suggested that the naphthoquinones act by inhibiting malarial electron transport (Fry and Pudney 1992; Hudson *et al.* 1985). In contrast, artemisinin and several 8-aminoquinolines inhibit malarial DHOD to the same or lesser extent than hypoxanthine uptake, suggesting that these drugs may have a different mode of action.

The assay procedure for DHOD described here differs from the assay described previously (Ittarat *et al.* 1992) in that it includes an internal standard and is therefore more quantitative. Tryptophan was found to be a good internal standard in this system because it is stable and inert and has a distinct elution time. This technique is sensitive, simple, and rapid and does not require radioactive substrate (Bennett *et al.* 1979; Kensler *et al.* 1981; Peters and Veerkamp 1984).

How does one determine whether a drug inhibits DHOD directly or causes a secondary decrease in enzyme activity as it kills the parasite by an alternate mechanism? We postulated that if a drug acts by inhibiting DHOD, then DHOD should be more sensitive to the drug than parasite viability. Parasite viability was measured using [³H]hypoxanthine uptake as an indicator of nucleic acid biosynthesis (Desjardins *et al.* 1979). Inhibitors of pyrimidine biosynthesis are particularly likely to inhibit nucleic acid synthesis since they should cause substrate depletion. However, if a drug inhibits nucleic acid synthesis without affecting DHOD activity, then it is likely that it has an alternate mode of action.

As a positive control, we tested atovaquone, a well-documented inhibitor of the parasite respiratory chain at the cytochrome bc 1 complex (Fry and Pudney 1992). As predicted, for a whole range of concentrations, atovaquone inhibits DHOD activity much more than [³H]hypoxanthine uptake. These data support the notion that

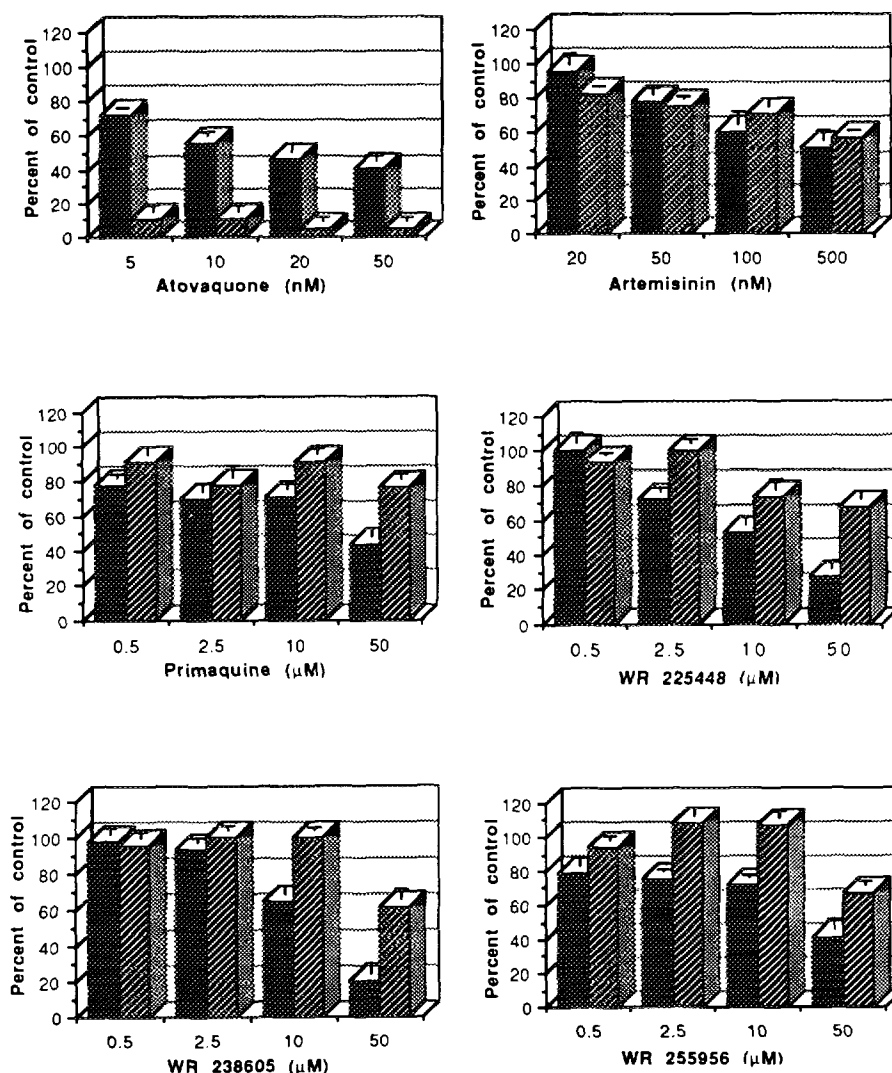


FIG. 3. The effects of drugs on the DHOD activity and hypoxanthine incorporation. Infected red cells were incubated in the presence of various concentrations of atovaquone, artemisinin, and 8-aminoquinolines, and assayed for DHOD activity (hatched bars) and hypoxanthine incorporation (solid bars), and expressed as a percentage of the DHOD activity of control untreated cultures. Data represent the means of triplicate readings \pm standard deviations.

one can determine whether a drug targets DHOD by comparing its effects on enzyme activity and hypoxanthine uptake.

We carried out the same tests on the 8-aminoquinolines, because there is evidence that these drugs also act by affecting plasmodial mitochondria. Two 8-aminoquinolines, primaquine and WR 225448, have

been shown to cause parasite mitochondrial swelling (Howells *et al.* 1970; Peters *et al.* 1984). Furthermore, radiolabeled primaquine has been shown to localize in the parasite mitochondrion (Aikawa and Beau-doin 1970). All of the 8-aminoquinolines tested inhibit hypoxanthine incorporation more than DHOD, suggesting that they do

not act by inhibiting this enzyme or electron transport.

Of particular interest is the observation that WR 255956 inhibits hypoxanthine incorporation more than DHOD activity. WR 255956 is a 5,6-dihydroxy-8-aminoquinoline derivative, which differs only in the side chain from those metabolites of primaquine which appear to be responsible for that drug's antimalarial activity (Bates *et al.* 1990). WR 255956 was studied instead of the authentic primaquine metabolites, since those compounds can no longer be obtained (W. Ellis, Walter Reed Army Institute of Research, personal communication). Because these primaquine metabolites are quinones, it has been suggested that they might inhibit mitochondrial electron transport in the same manner as the naphthoquinones (Warhurst 1984). However, the data presented here for WR 255956 suggest that this is not the case. The difference between the 5,6-dihydroxy-8-aminoquinolines and the 2-hydroxynaphthoquinones may be related to the fact that they have very different redox potentials (Lopez-Shirley *et al.* 1994).

The effects of artemisinin on DHOD were studied because of electron microscopic evidence that artemisinin derivatives affect parasite mitochondria. Dihydroartemisinin and artemisinin have also been shown to localize in plasmodial mitochondria (Ellis *et al.* 1985; Maeno *et al.* 1993) and to cause mitochondrial swelling (Ellis *et al.* 1985; Jiang *et al.* 1985). Furthermore, the possibility existed that artemisinin might be alkylating mitochondrial cytochromes, since artemisinin forms covalent adducts with heme (Meshnick *et al.* 1991; Hong *et al.* 1994). The data presented here suggest that artemisinin does not inhibit mitochondrial electron transport in the same way as atovaquone.

ACKNOWLEDGMENTS

This work was supported by grants from NIH (AI26848) and the World Bank/UNDP/World Health

Organization Special Programme for Research and Training in Tropical Diseases. We thank Mr. William Ellis, Walter Reed Army Institute of Research, and Dr. Michael Rogers, Burroughs Wellcome, for supplying compounds.

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Received 16 February 1994; accepted with revision 26 April 1994