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Effects of Atovaquone and Other Inhibitors on *Pneumocystis carinii* Dihydroorotate Dehydrogenase

ISRA ITTARAT,[1] WANIDA ASAWAMAHASAKDA,[1]† MARILYN S. BARTLETT,[2] JAMES W. SMITH,[2] AND STEVEN R. MESHNICK[1]*

Department of Epidemiology, University of Michigan School of Public Health, Ann Arbor, Michigan,[1] and Department of Pathology and Laboratory Medicine, Indiana University Medical Center, Indianapolis, Indiana[2]

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Dihydroorotate dehydrogenase (DHOD) is a pyrimidine biosynthetic enzyme which is usually directly linked to the mitochondrial respiratory chain. Antimalarial naphthoquinones such as atovaquone (566c80) inhibit malarial DHOD by inhibiting electron transport. Since atovaquone also has therapeutic activity against *Pneumocystis carinii*, the *P. carinii* DHOD may also be an important drug target. Organisms were obtained from immunosuppressed rats, incubated for 24 h in a short-term in vitro culture system, and then lysed. *P. carinii* lysates catalyzed the generation of orotate from dihydroorotate at a rate of 852 pmol/mg of protein per min. Control preparations made from uninfected mice showed much less total enzymatic activity and enzyme specific activity. As expected, *P. carinii* DHOD activity was susceptible to respiratory inhibitors such as cyanide, antimycin A, and salicylhydroxamic acid (SHAM). Susceptibility to SHAM suggests the presence of an alternative oxidase. In contrast, neither pentamidine nor 5-hydroxy-6-demethylprimaquine (5H6DP), a quinone metabolite of primaquine, inhibited the enzyme. Atovaquone inhibited DHOD by 76.3% at 100 μM and 36.5% at 10 μM. A similar degree of inhibition was found when the organisms were preincubated with the drug. Atovaquone inhibited *P. carinii* growth in vitro at a somewhat lower concentration (between 0.3 and 3 μM). In contrast, *Plasmodium falciparum* growth and enzyme activity are susceptible to nanomolar concentrations of atovaquone. Thus, while it is possible that atovaquone acts by inhibiting the *P. carinii* electron transport chain, the possibility of another drug target cannot be excluded.

Dihydroorotate dehydrogenase (DHOD) is a key enzyme in de novo pyrimidine biosynthesis, catalyzing the conversion of dihydroorotate to orotate (5, 12, 22). DHOD is a particularly important enzyme, because it is the target of several useful chemotherapeutic agents such as the antitumor agent brequinar sodium (6) and the antimalarial agents atovaquone and menoctone (13, 17). Several orotate analogs have antimalarial activities (20, 25). Since atovaquone is also an effective treatment for *Pneumocystis carinii* pneumonia (2), it is possible that the *P. carinii* DHOD might be an important chemotherapeutic target. But little is currently known about the *P. carinii* DHOD.

In all eukaryotic cells that have been studied except trypanosomatids (23), DHOD is bound to mitochondria. Isolated enzymes donate electrons directly to oxygen and to various artificial electron acceptors, although they usually prefer coenzyme Q (ubiquinone) (22). In intact organisms, carefully broken cells, and isolated mitochondria, most DHODs donate electrons directly to the mitochondrial respiratory chain.

In a previous study we have examined the effects of various drugs on the mitochondrion-bound DHOD of *Plasmodium falciparum* (18). We then looked at the effects of these and other drugs on the *P. carinii* DHOD, which are described here.

* Corresponding author. Mailing address: Department of Epidemiology, University of Michigan School of Public Health, 109 Observatory St., Ann Arbor, MI 48109-2029. Phone: (313) 747-2406. Fax: (313) 764-3192. Electronic mail address: MESHNICK@UMICH.EDU.

† Present address: Department of Clinical Microscopy, Faculty of Medical Technology, Mahidol University, Siriraj Hospital, Bangkok 10700, Thailand.

MATERIALS AND METHODS

Drugs. Atovaquone (566c80) was a gift from the Burroughs Wellcome Co., Research Triangle Park, N.C. 5-Hydroxy-6-demethylprimaquine (5H6DP) was a gift from Mohamed Nasr, Division of AIDS, National Institute of Allergy and Infectious Diseases, Bethesda, Md. All other chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise noted.

Isolation of organisms for enzyme assay. Barrier-raised, not certified pathogen-free Sprague-Dawley rats (Hilltop Laboratories, Scarsdale, Pa.) were administered dexamethasone (2 mg/liter; Vedco, St. Joseph, Mo.) and tetracycline (0.5 g/liter) in their drinking water and were fed low-protein (8%) chow (Ziegler Brothers, Gardners, Pa.) by the method of Tidwell et al. (26). After 8 to 12 weeks, the rats were sacrificed and their lungs were removed, washed with Dulbecco's phosphate-buffered saline (PBS), minced with a razor, and treated with collagenase (6 mg of enzyme per 20 ml of PBS) at 37°C in a shaking water bath for 30 min. The homogenate was pressed through a wire mesh screen and was centrifuged at 1,000 × *g* for 10 min at 4°C. The resulting pellet was suspended in 10 ml of 0.85% ammonium chloride, and the suspension was incubated at 37°C for 5 min, after which it was passed through a polycarbonate filter with 8-μm pores (Fisher Scientific, Pittsburgh, Pa.). The filtrate was then centrifuged at 1,000 × *g* and washed twice with PBS. Slides were prepared, fixed, and stained with Giemsa (Harleco), Gram, and cresyl violet stains. The preparations contained numerous trophozoites and cysts but no bacteria or fungi.

Control preparations from the lungs of nonimmunosuppressed rats were made in an identical manner.

Short-term cultivation of organisms. Freshly harvested organisms were then incubated in the presence of human embryonic lung (HEL) cells (10). HEL cells were grown to near confluence in 24-well microtiter plates in Eagle's minimal essential medium containing 10% heat-inactivated fetal calf serum at 37°C in a 5% CO_2 atmosphere. Lung-derived organisms (or control preparations from uninfected lungs) were added to the wells of the microtiter plates (approximately 140 μg of protein per well), and the plates were incubated for 24 h prior to all experiments. In some experiments, drugs were added to the microtiter wells after 20 h of incubation and were then incubated for an additional 4 h.

Analysis of DHOD. Organisms were dislodged from the feeder cells by repeated pipetting, washed with Dulbecco's PBS, and lysed by three cycles of freezing and thawing. Cultures from uninfected rat lungs were treated in the same manner. DHOD activity was assayed by a high-performance liquid chromatography method described previously (18). Protein was determined by the D_C Protein Assay (Bio-Rad, Rockville Center, N.Y.), with bovine serum albumin used as a standard.

Inhibition of *P. carinii* growth in vitro. In vitro drug inhibition was determined as described previously (3). Briefly, HEL cells were plated to confluence in

TABLE 1. Effects of respiratory inhibitors on *P. carinii* DHOD activity

Inhibitor	Concn	% Inhibition (no.)[a]
KCN	100 μM	58.3 ± 17 (3)
	1 mM	73.3 ± 16.5 (4)
Thiobarbiturate	1 mM	16.9 ± 3.5 (2)
SHAM	100 μM	−7.4 ± 13.4 (3)
	1 mM	80.3 ± 11.8 (2)
Antimycin A	10 μM	46.7 ± 14.4 (4)
	100 μM	69.3 ± 8.0 (4)

[a] Values are means ± standard deviations.

TABLE 2. Effects of chemotherapeutic agents on *P. carinii* DHOD activity

Inhibitor	Concn (μM)	% Inhibition (no.)[a]
Atovaquone	1	0.7 ± 1.0 (2)
	10	36.5 ± 6.2 (4)
	100	76.3 ± 5.2 (4)
Pentamidine	10	−15.7 ± 2.3 (2)
5H6DP	10	−3.9 ± 10.8 (2)
	100	−11.9 ± 17.5 (2)

[a] Values are means ± standard deviations.

Eagle's minimum essential medium with 10% fetal calf serum in 24-well microtiter plates. Each well was inoculated with *P. carinii* from the lungs of a transtracheally inoculated infected rat for a final concentration of about 7×10^5 viable organisms per well. Drugs were incorporated into tissue culture medium so that there were four untreated wells per parameter per time point, and each plate included untreated and trimethoprim-sulfamethoxasole-treated (50 and 250 μg/ml, respectively) controls. Plates were incubated at 35°C in 5% O_2–10% CO_2–85% N_2 for 7 days. Organisms were dislodged by pipetting, and 10-μl samples were taken on days 1, 3, 5, and 7, transferred to a 1-by-1-cm square etched on a glass slide, stained with Giemsa (Harleco), and examined as unknowns by two microscopists. The numbers of organisms in 10 randomly selected ×1,000-magnification fields were averaged for each well sample. Scores are averages of eight values (four wells per parameter × two examiners).

RESULTS

P. carinii preparations made from freshly harvested rat lungs contained a substantial amount of DHOD activity. However, the DHOD activities of freshly prepared control preparations (filtrates from uninfected lungs) were also quite high (data not shown) and may have been contaminated with rat lung mitochondria. In order to eliminate this artifact, filtrates from infected lungs (as well as uninfected control lungs) were incubated in culture for 24 h in the presence of HEL cells prior to measuring enzyme activity. After this treatment, preparations from uninfected rat lungs contained only approximately 1% of the total enzyme activity found in the preparations from infected lungs. Thus, from each uninfected rat preparation, the total recovered enzyme activity was only 0.146 ± 0.053 nmol/min ($n = 3$), compared with 13.49 ± 0.98 nmol/min from each infected rat preparation ($n = 5$).

The specific activity of DHOD found in *P. carinii* lysates was also higher than the specific activity found in control preparations. The specific activity of DHOD found in *P. carinii* lysates was 852 ± 67 pmol/mg of protein per min ($n = 5$). This is similar to the specific activities of DHOD reported for *P. falciparum* (400 to 1,600 pmol/mg of protein per min, depending on the stage) (19). The specific activity of the control preparations made from uninfected rat lungs, 169 ± 61 pmol/mg of protein per min ($n = 3$), was substantially lower. Thus, there is relatively little contamination of the *P. carinii* lysates by host-derived material.

Since DHOD donates its electrons to the respiratory chain via ubiquinone, we next evaluated the effects of a series of inhibitors of respiration (Table 1). *P. carinii* DHOD activity, as expected, could largely be inhibited by KCN, which inhibits cytochrome oxidase (complex IV), and antimycin A, which inhibits site 2 (complex III), both of which are downstream from ubiquinone (21). Not surprisingly, thiobarbiturate, which inhibits upstream of ubiquinone at site I (between complex I and ubiquinone), did not cause significant inhibition, which is consistent with previous observations that DHOD donates electrons directly to ubiquinone. Finally, the *P. carinii* DHOD was inhibited by salicylhydroxamic acid (SHAM; 1 mM), which also inhibits downstream from ubiquinone at the alternative oxidase (9).

We next evaluated the effects of chemotherapeutic agents on the *P. carinii* DHOD (Table 2). Atovaquone was found to inhibit *P. carinii* DHOD activity at concentrations of 10 and 100 μM. 5H6DP, a quinone metabolite of primaquine, was also evaluated since this compound has been hypothesized to be a DHOD inhibitor (27). No effect was observed at 10 or 100 μM 5H6DP. As a control, we evaluated the effects of pentamidine, which is known to have targets other than DHOD (11), to determine whether the observed inhibition is specific or nonspecific. No inhibition of DHOD by pentamidine was observed.

The relative insusceptibility of *P. carinii* to atovaquone in comparison with that of *P. falciparum* (13, 18) might have been due to the inability of the drug to be taken up by mitochondria during the assay. Accordingly, organisms were preincubated with atovaquone for 4 h prior to assaying drug activity. Atovaquone was only slightly more effective in this manner, inhibiting *P. carinii* DHOD activity by 0% ($n = 1$), 59% ± 18% ($n = 3$), and 88.1% ± 10.1% ($n = 4$) at 1, 10, and 100 μM, respectively. A similar experiment was performed with 5H6DP. No inhibition was seen in organisms preincubated in 10 or 100 μM 5H6DP (−6.5% ± 5.4% [$n = 2$] and 2.3% ± 9.6% [$n = 3$], respectively).

In order to confirm that *P. carinii* is less susceptible to atovaquone than malaria parasites, the effects of atovaquone on parasite growth in vitro were assessed (Fig. 1). Almost complete inhibition of growth was achieved in the presence of 30 and 3 μM drug, while no inhibition of growth was seen in the presence of 0.3 and 0.03 μM drug.

DISCUSSION

P. carinii contains a DHOD which appears to be linked to the mitochondrial electron transport chain, as it is in most other eukaryotes. Consistent with this, *P. carinii* DHOD activity is susceptible to antimycin A, which inhibits complex III, and cyanide, which inhibits complex IV, both of which are downstream from ubiquinone (20), which is where DHOD is believed to donate electrons (5, 12, 22).

It is unlikely that host-derived DHOD activity contributed in any important fashion to the DHOD activity measured in *P. carinii* lysates. Control lysates (made from sham cultures prepared from uninfected rat lungs) contained only approximately 1% of the DHOD activity found in *P. carinii* lysates. One of the reasons that this contamination is so low is that most of the rat

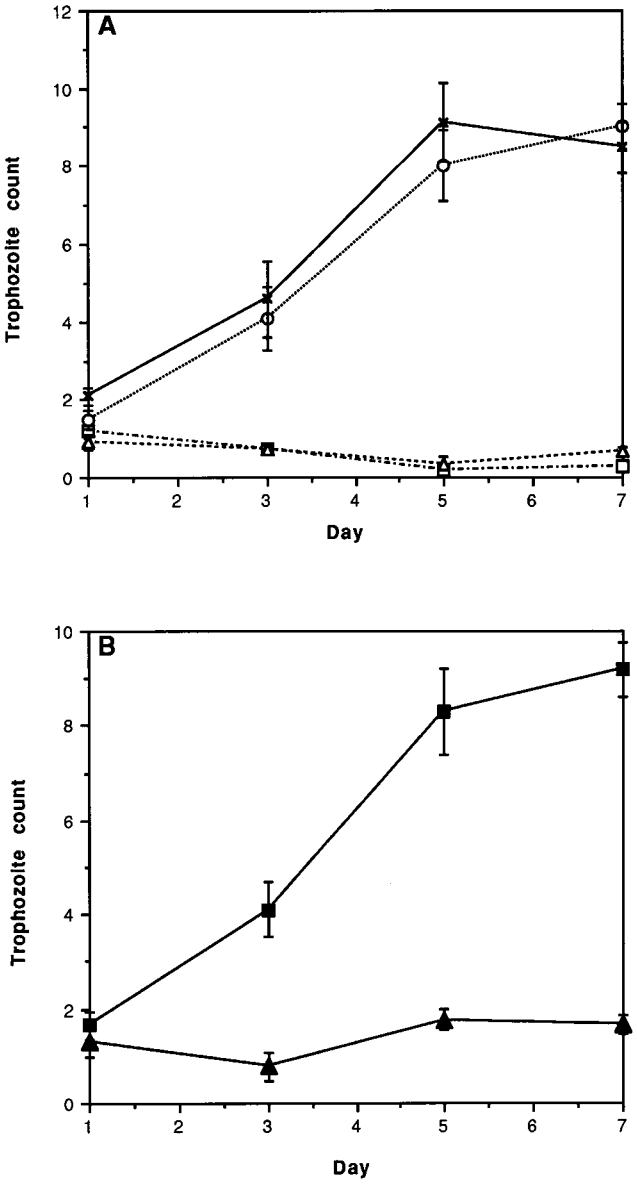

FIG. 1. Growth of *P. carinii* in vitro in the presence of atovaquone (A) at 0.03 μM (×), 0.3 μM (○), 3.0 μM (△), and 30 μM (□) and, as controls (B), in the absence of drug (■) and in the presence of trimethoprim-sulfamethoxasole (▲). Data are means ± standard deviations.

lung mitochondria appear to become inactive after 24-h incubations in vitro. Of course, uninfected rat lung tissue is different from the lung tissues of rats suffering from *P. carinii* infection, particularly in the quantity of inflammatory cells present. However, it is unlikely that, even with these inflammatory cells, there could have been more than a two- to threefold increase in the quantity of contaminating rat lung mitochondria. Thus, it is unlikely that these rat-derived mitochondria could have contributed to the measured susceptibilities to the inhibitors (Tables 1 and 2).

SHAM is a well-known inhibitor of the alternative oxidases of plants, protozoa, and fungi (9). The observed pattern of susceptibility to SHAM is unusual, in that nearly full inhibition occurs at 1 mM, while no inhibition was detected at 100 μM, and also that the sum of the effects of cyanide and SHAM is greater than 100%. Thus, the data presented here suggest but do not clearly demonstrate the presence of an alternative oxidase in *P. carinii*.

It was somewhat surprising that atovaquone only inhibited *P. carinii* DHOD at concentrations of ≥10 μM. This concentration is >10,000-fold higher than the concentration which inhibits malarial DHOD (~1 nM) (13). There was a similar difference in the inhibitory effects of the drug on parasite growth. On the basis of the data presented here, the 50% inhibitory concentration for *P. carinii* growth is between 0.3 and 3 μM, which is also substantially higher than the reported 50% inhibitory concentration for *P. falciparum* (also ~1 nM) (8, 15). Nevertheless, the concentrations which inhibit *P. carinii* growth and DHOD are similar to or lower than the mean concentrations in serum (25 to 50 μM) which have been measured in patients receiving therapeutic doses of the drug (2).

Primaquine and other 8-aminoquinolines have been found to be highly effective against *P. carinii* (24). Primaquine is metabolized into quinones by the liver (28), and these quinone metabolites probably account for the drug's antimalarial activity (4). It was hypothesized that the metabolites of the quinone primaquine act by inhibiting DHOD (27). We have recently reported that this is not true for *P. falciparum* (18). We found in the present study that, as in malaria, 5H6DP does not inhibit *P. carinii* DHOD (Table 2). Interestingly, the modes of action of the 8-aminoquinonlines may be different in malaria parasites and *P. carinii*. Primaquine-treated malaria parasites show morphological changes in their mitochondria (1, 16), an effect which is not seen in *P. carinii* (14). Thus, the mode of antipneumocystis action of the 8-aminoquinolines still needs to be elucidated.

In the present study, we have shown that atovaquone inhibits *P. carinii* DHOD activity at concentrations which are achievable in vivo. However, there are still three possibilities for its mode of antipneumocystis action. First, it might kill the organisms by causing pyrimidine depletion as it does in malaria parasites. Second, by inhibiting electron transport in *P. carinii*, atovaquone might kill the organisms by depleting their ATP. Third, atovaquone might have an entirely different target. Further work is needed to determine which of these possibilities is correct.

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