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ANTIMALARIAL ACTIVITY OF OROTATE ANALOGS THAT INHIBIT DIHYDROOROTASE AND DIHYDROOROTATE DEHYDROGENASE

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Abstract—Dihydroorotase and dihydroorotate dehydrogenase, two enzymes of the pyrimidine biosynthetic pathway, were purified from *Plasmodium berghei* to apparent homogeneity. Orotate and a series of 5-substituted derivatives were found to inhibit competitively the purified enzymes from the malaria parasite. The order of effectiveness as inhibitors on pyrimidine ring cleavage reaction for dihydroorotase was 5-fluoro orotate > 5-amino orotate, 5-methyl orotate > orotate > 5-bromo orotate > 5-iodo orotate with K_i values of 65, 142, 166, 860, 2200 and >3500 μM, respectively. 5-Fluoro orotate and orotate were the most effective inhibitors for dihydroorotate dehydrogenase. *In vitro*, 5-fluoro orotate and 5-amino orotate caused 50% inhibition of the growth of *P. falciparum* at concentrations of 10 nM and 1 μM, respectively. In mice infected with *P. berghei*, these two orotate analogs at a dose of 25 mg/kg body weight eliminated parasitemia after a 4-day treatment, an effect comparable to that of the same dose of chloroquine. The infected mice treated with 5-fluoro orotate at a lower dose of 2.5 mg/kg had a 95% reduction in parasitemia. The effects of the more potent compounds tested in combination with inhibitors of other enzymes of this pathway on *P. falciparum in vitro* and *P. berghei in vivo* are currently under investigation. These results suggest that the pyrimidine biosynthetic pathway in the malarial parasite may be a target for the design of antimalarial drugs.

The spread of drug-resistant malaria accompanied by a world-wide resurgence of the disease requires the development of new chemotherapeutic agents for the management of malaria. According to World Health Organization estimates of 1989, 100 million people were infected with malaria which makes it the most widespread and devastating of the tropical diseases, causing 2 million deaths per year [1].

Malaria parasites require purines and pyrimidines for DNA and RNA synthesis during exponential growth. The parasites use preformed purines which are salvaged from the host, but they have to synthesize pyrimidine *de novo* [2–6]. Several lines of evidence suggested that there are some key differences between malarial parasites and higher eukaryotes. The first three enzymes of the pyrimidine pathway, carbamyl phosphate synthase II (CPS II†; EC 6.3.5.5), aspartate transcarbamylase (ATCase; EC 2.1.3.2), and dihydroorotase (DHOase; EC 3.5.2.3), are carried by a M_r 240,000 multifunctional protein [7, 8] in most eukaryotes, whereas in malaria parasites they exist as separate proteins [4]. The fourth enzyme of the pathway, dihydroorotate dehydrogenase (DHODase; EC 1.3.3.1), has been characterized in the malaria parasites [9] and found to be different from that in host mammalian cells [7, 8]. The last two enzymes, orotate phosphoribosyltransferase (OPRTase; EC 2.4.2.10) and orotidine 5′-phosphate decarboxylase (ODCase; EC 4.1.1.23), catalyze the synthesis of UMP in host mammalian cells and exist as a bifunctional protein [8], but in malaria parasites these proteins have been described as discrete entities [10]. Finally, host mammalian cells can either use preformed pyrimidine base and nucleosides or synthesize their own [8, 11], while, in contrast, the malaria parasites are totally dependent on *de novo* synthesis for pyrimidine requirements because they lack salvage enzymes, notably, thymidine kinase [3, 5, 6].

Recently, it was found that 5-fluoro orotate, an orotate analog, has potent antimalarial activity both *in vitro* against *Plasmodium falciparum* [11, 12] and *in vivo* against *P. yoelii* in mice [13]. These results suggest that the malaria parasite may be sensitive to novel drugs that act on pyrimidine *de novo* synthesis. Therefore, we have focused on the metabolism of the parasite and the mechanism of action of 5-substituted orotate analogs. In this study, we directly tested 5-fluoro orotate and other orotate analogs against at least two enzymes of the pyrimidine biosynthetic pathway purified from the mouse malaria parasite *P. berghei*, and measured *in vitro* antimalarial activity of the orotate analogs on *P. falciparum*; we also tested their effects *in vivo* against mice infected with *P. berghei*.

MATERIALS AND METHODS

Materials. Potassium [^{14}C]cyanate (56.7 Ci/mol)

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† Abbreviations: ATCase, aspartate transcarbamylase; CPS II, carbamyl phosphate synthase II; L-CA, L-carbamylaspartate; DCIP, dichlorophenol-indophenol; L-DHO, L-dihydroorotate; DHOase, dihydroorotase; DHODase, dihydroorotate dehydrogenase; FPLC, fast protein liquid chromatography; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; LDH, lactate dehydrogenase; Me_2SO, dimethyl sulfoxide; L-OA, orotate; OPRTase, orotate phosphoribosyltransferase; PMSF, phenylmethylsulfonyl fluoride; PEI-cellulose, poly-(ethyleneimine)-cellulose.

and [*carboxyl*-^{14}C]orotate (52.5 Ci/mol) were purchased from Du Pont-NEN. The chromatographic technique was carried out on a fast protein liquid chromatographic system (FPLC) purchased from Pharmacia LKB. Cibacron Blue F3GA-agarose affinity gel, L-carbamylaspartate (L-CA), L-dihydroorotate (L-DHO), and orotate (L-OA) and its 5-substituted derivatives were purchased from Sigma. All other chemicals, reagents and materials were of the highest quality commercially available and were used without further purification.

L-[^{14}C]Carbamylaspartate (L-[^{14}C]CA) was synthesized from potassium [^{14}C]cyanate and L-aspartate as described [14]. The radiochemical purity of L-[^{14}C]CA was 98.6%, as determined by TLC on poly(ethyleneimine)-cellulose (PEI-cellulose) [14], and it had specific radioactivity of 143.5 Ci/mol determined by using a colorimetric method as described previously [15].

[*carboxyl*-^{14}C]Dihydroorotate (L-[^{14}C]DHO) was synthesized from [*carboxyl*-^{14}C]orotate as described [16], and purified by FPLC on a Mono Q anion-exchange column with 0.2 M LiCl as eluent. The radiochemical purity of L-[^{14}C]DHO was >99%, as determined by TLC on PEI-cellulose [14], and had specific radioactivity of 52.2 Ci/mol.

Parasite culture. P. falciparum (K_1 isolate) was cultured in RPMI (Gibco) with 25 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES), 32 mM $NaHCO_3$ and 10% human serum by a modified method of Trager and Jensen [17]. The red cell (group 0) suspension was used at 5% hematocrit. Synchrony was maintained by the sorbitol procedure of Lambros and Vanderberg [18]. *P. berghei* was cultivated in Balb/C mice. Cell-free extracts of the parasites were prepared as described previously [19] in the presence of the following protease inhibitors: 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EDTA and 0.03 mg/mL of leupeptin, pepstatin and *N*-*p*-tosyl-L-lysine chloromethyl ketone.

Enzyme purification. DHOase was purified from *P. berghei* to apparent homogeneity by following FPLC on a Mono Q anion-exchange column, a phenyl-superose column, a Mono P chromatofocusing column and finally a gel filtration Superose 12 column according to Krungkrai *et al.* [4]. The enzyme was stored at 4° and stabilized by 30% dimethyl sulfoxide (Me_2SO). For DHODase enzyme, following Triton X-100 solubilization of the parasite membrane, chromatography on Mono Q, Cibacron Blue F3GA-agarose affinity and finally gel filtration Superose 12 columns, the malarial DHODase was purified to apparent homogeneity as described recently [9].

Enzyme assays. DHOase activity was measured in the ring cleavage (L-DHO → L-CA) and cyclization reaction (L-CA → L-DHO) as follows: The assay mixture contained 50 mM HEPES (pH 7.4), 15% Me_2SO, 0.25 mM L-[^{14}C]DHO or L-[^{14}C]CA, and enzyme in a total volume 0.02 mL. The reaction mixtures were incubated at 37° for 15 min, and product formation was quantified by TLC [14]. DHODase activity was assayed according to Karibian and Couchoud [20]. In a typical assay, ubiquinone-30 was used as the final electron acceptor. The reaction mixture contained 0.25 mM L-DHO, 0.07 mM ubiquinone-30, 0.045 mM dichlorophenol-indophenol (DCIP) and enzyme in 100 mM Tris–HCl (pH 8.0). The reaction was monitored at 37° by the loss of DCIP absorbance at 600 nm with an extinction coefficient of 21,500 M^{-1} cm^{-1}.

In vitro *antimalarial test.* The parasite growth during drug-screening tests was quantitated by measuring [^{3}H]hypoxanthine incorporation [21] and parasitemia based on morphological observations [22]. Various concentrations of test compound solutions dispensed in 25 μL of the culture medium were added into individual wells of 96-well microtiter plates. Two hundred microliters of red cell suspensions at 2% hematocrit with 0.5% parasitemia was added to each well. The plates were incubated at 37° for 24 hr in a candle jar atmosphere [17]. [^{3}H]-Hypoxanthine (0.5 μCi; 1 Ci/mol) in 25 μL of the culture medium was then added to each well. The incorporation of [^{3}H]hypoxanthine in each well was examined after 48 hr of drug-treated culture as described earlier [21]. Work by others has shown that [^{3}H]hypoxanthine incorporation is a reliable measure of parasite growth and development [23]. In addition, parasite morphology in drug-treated culture after 96 hr was measured by staining with Giemsa. All compounds were run in triplicate at each concentration. Drug-free control cultures were run simultaneously. All data points represent means of at least three experimental tests. The 50 and 90% inhibitory concentrations (IC_{50} and IC_{90}) are defined, respectively, as the drug concentration inhibiting [^{3}H]hypoxanthine incorporation by 50% at 48 hr and the drug concentration causing 90% inhibition of parasitemia, observed by examining the morphology of the parasite at 96 hr, compared with drug-free controls incubated under the same conditions.

Antimalarial test against P. berghei in vivo. A 4-day suppressive test of blood schizontocidal action against *P. berghei* in female Balb/C mice (25 ± 2 g) was conducted by daily intraperitoneal injections for 4 days with chloroquine and some orotate analogs at doses of 2.5, 10 and 25 mg/kg body weight starting from the day of parasite inoculation (1×10^7 parasites). Thin blood films were prepared daily for morphological examination under a light microscope. At least 500 red cells were examined in each slide. Each result shown represents the mean ± SD from six mice.

Cytotoxicity test. The cytotoxic effect of the 5-substituted orotate derivatives was quantitated by assaying lactate dehydrogenase (LDH) in macrophages. The macrophages were obtained from peritoneal exudates of mice. After removal of nonadherent cells, cells were cultured overnight at a density of 2×10^6/mL in Dulbecco's Modified Eagle's Medium containing 10% heat-inactivated fetal bovine serum. The same medium supplemented with increasing concentrations of the orotate analogs was then added and incubation continued for 48 hr in a humidified CO_2 incubator. At the end of the incubation the cells were washed, lysed, and assayed for LDH activity.

Other methods. Sodium dodecyl sulfate–poly-

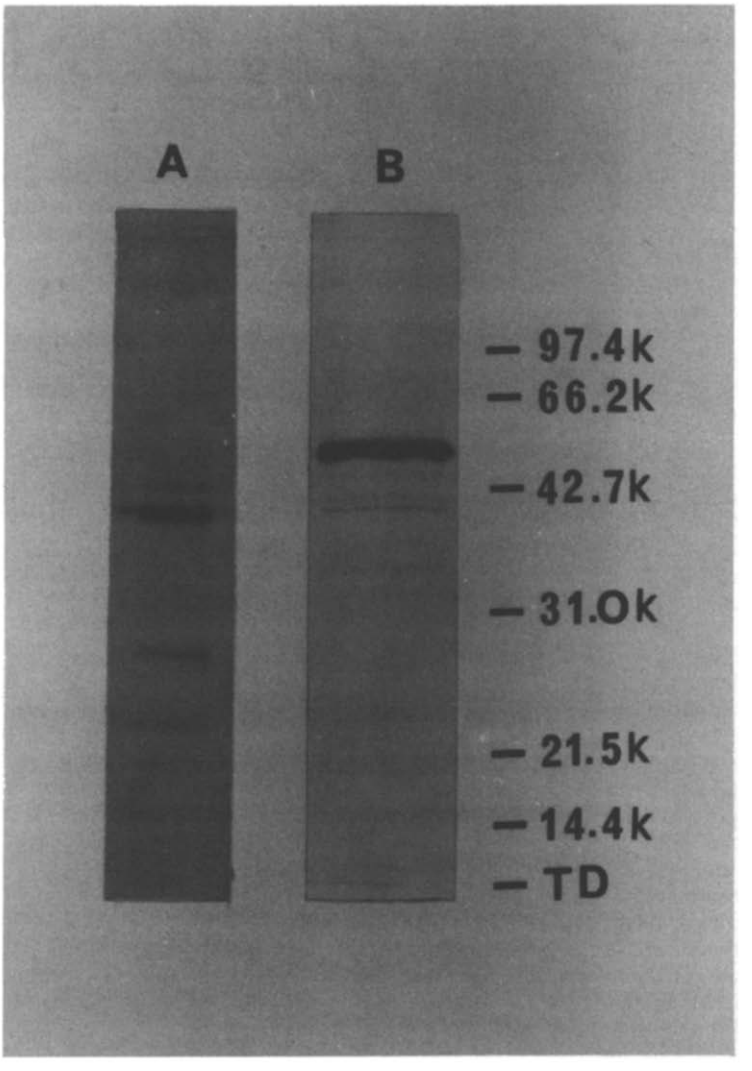

Fig. 1. SDS–PAGE analysis of purified DHOase (A) and DHODase (B) from *P. berghei*. A 10% polyacrylamide gel was run in 0.1% SDS according to the method of Laemmli [24]. Marker proteins were phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (42.7 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa) and TD (bromphenol blue) as tracking dye. The purified DHOase (3 μg) and DHODase (8 μg) were first stained with Coomassie blue and then with silver.

acrylamide gel electrophoresis (SDS–PAGE) was performed on a Bio-Rad minislab gel apparatus with a 5% acrylamide stacking gel and a 10% acrylamide running gel in the discontinuous buffer system of Laemmli [24]. The apparent inhibition constant (K_i) values of the test compounds were determined from Dixon plots of kinetic data obtained by varying concentrations of the inhibitors and these were fitted with least-squares analysis. Protein concentrations were determined by the method of Bradford [25] with bovine serum albumin as standard. Aqueous solutions of ubiquinone-30 were prepared by dissolving the compound in 1% Triton X-100.

RESULTS

Purification of malarial dihydroorotase and dihydroorotate dehydrogenase. The *P. berghei* DHOase and DHODase were purified essentially by the procedure described by Krungkrai *et al.* [4, 9]. The purity of the DHOase and DHODase was assessed by SDS–PAGE (Fig. 1). The purified DHOase and DHODase appeared as single bands when visualized by Coomassie blue and silver stainings with molecular weights of 38 and 52 K, respectively.

Inhibition of dihydroorotase by orotate analogs. Using 15% Me_2SO as an enzyme stabilizer, this approach allowed us to investigate the kinetic parameters for the purified DHOase on both ring cyclization (L-CA → L-DHO) and cleavage (L-DHO → L-CA) reactions. The K_m and k_{cat} values for the ring cyclization reaction were 340 μM and 207 min^{-1}, respectively. For the ring cleavage reaction the K_m and k_{cat} values were 18.5 μM and

Table 1. Kinetic constants of L-CA, L-DHO, L-OA and its analogs in ring cyclization and cleavage reactions of *P. berghei* DHOase at pH 7.4 in the presence of 15% Me_2SO

Substrates or orotate analogs	Diameter of substituent in position 5 (Å)	Ring cyclization			Ring cleavage		
		K_m (μM)	k_{cat} (min^{-1})	K_i (μM)	K_m (μM)	k_{cat} (min^{-1})	K_i (μM)
L-CA		340	207				
L-DHO					18.5	188	
L-OA				630			860
F-OA	2.7			125			65
NH_2-OA	2.9			395			142
CH_3-OA	3.5			480			166
Br-OA	3.8			1250			2200
I-OA	4.1			>3500			>3500

188 min^{-1}, respectively. The effects of orotate and its 5-substituted analogs were tested on both reactions of malarial DHOase. All of the six compounds tested were competitive inhibitors of the enzyme. The K_i values obtained from Lineweaver–Burk plots and also Dixon plots of the ring cyclization and cleavage reactions were tabulated and are shown in Table 1. The most effective inhibitor of both reactions was 5-fluoro orotate. Interestingly, 5-fluoro, 5-amino, and 5-methyl orotate were found to be more effective inhibitors than orotate itself in both reactions. 5-Bromo and 5-iodo orotates were much less effective inhibitors than orotate. Increasing K_i values of the 5-substituted orotate analogs of the malarial DHOase were correlated to the increase in the size of the 5 substituents as also found with the mammalian enzyme by Christopherson and Jones [26]. However, some kinetic values for the malarial enzyme were generally similar, although much higher or lower than those reported for the mammalian [7, 26] and the bacterial systems [27], respectively.

Inhibition of dihydroorotate dehydrogenase by orotate analogs. The K_m for L-DHO and the k_{cat} values of the purified DHODase were 8 ± 2 μM and 11.5 min^{-1}, respectively. This enzyme had no orotate reductase catalyzing L-OA → L-DHO. It was found that orotate, the product of the enzyme reaction, was an effective competitive inhibitor with a K_i value of 31 μM (Fig. 2). All of the 5-substituted orotate analogs tested against the malarial DHODase were found to be competitive inhibitors. Using Dixon plots the apparent K_i values of these orotate analogs were obtained. As shown in Table 2, the K_i values of 5-fluoro and 5-amino orotate were comparable to the K_i of orotate itself. 5-Bromo and 5-iodo orotate were less effective inhibitors. The results on 5-amino, 5-bromo and 5-iodo orotate of the malarial DHODase were slightly different from those reported with bovine [28] and rat liver [29] enzymes.

Antimalarial activity on P. falciparum *of orotate analogs* in vivo. The effects of various concentrations of the 5-substituted orotate analogs on parasite growth for 48 hr as measured by [^{3}H]hypoxanthine uptake are presented in Fig. 3. The IC_{50} values were determined as the concentration of the tested compound inhibiting hypoxanthine incorporation by 50%. It was found that 5-fluoro orotate had the most

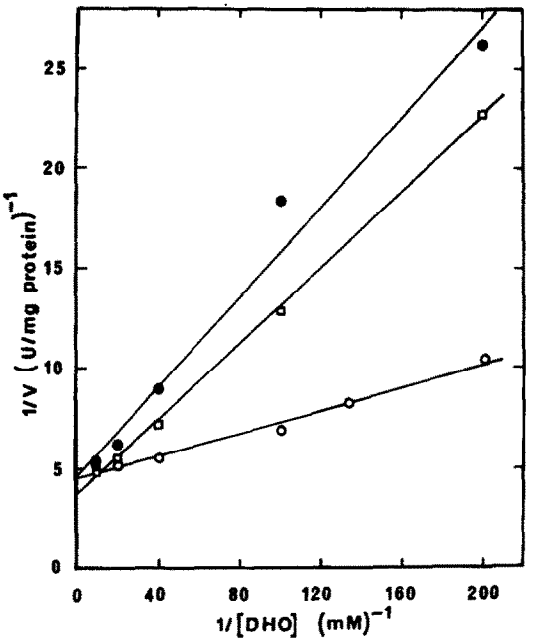

Fig. 2. Lineweaver–Burk plots of the inhibitory effects of L-OA and 5-fluoro orotate on malarial DHODase activity. All assays were conducted at 37° and in 100 mM Tris–HCl, pH 8.0. The concentrations of inhibitors used were 100 μM 5-fluoro orotate (●), and 100 μM L-OA (□); the control contained no inhibitors (○).

potent antimalarial activity. The IC_{50} values for 5-fluoro, 5-methyl and 5-amino orotates were 10 nM, 1 μM and 10 μM, respectively. Orotate, 5-bromo and 5-iodo orotate at concentrations as high as 1.0 mM showed no effect on parasite growth. Based on parasite morphological examination of 96-hr treatment (for at least two intraerythrocytic cycles) with the orotate analogs, the IC_{90} values (concentrations of the compounds having 90% inhibition of growth determined by examining the morphology of the parasite) of the effective orotate analogs were tabulated and are presented in Table 2.

Table 2. Kinetic and inhibition constants for *P. berghei* DHODase and antimalarial activity of orotate analogs against *P. falciparum* growth *in vitro*

Compounds	K_m (μM)	K_i (μM)	IC_{90}^{*} (μM)
Substrate			
L-DHO	8 ± 2		
Inhibitor			
L-OA		31	NE†
F-OA		35	0.06
NH_2-OA		44	50.0
CH_3-OA		158	6.0
Br-OA		380	NE
I-OA		464	NE

* Concentration of compound producing 90% inhibition of *P. falciparum* growth *in vitro*.

† No effect on parasite morphology at 1.0 mM.

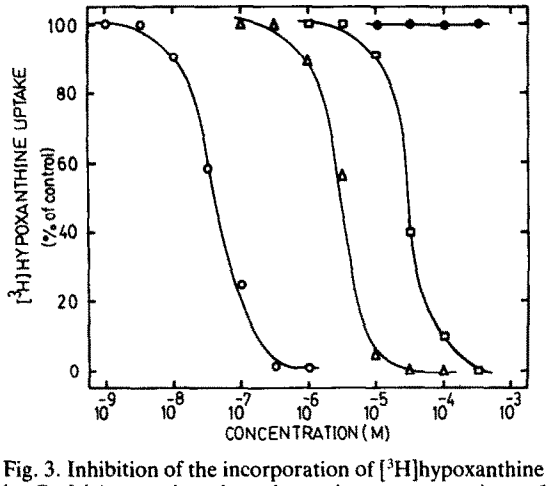

Fig. 3. Inhibition of the incorporation of [^{3}H]hypoxanthine by *P. falciparum* in culture by various concentrations of the 5-substituted orotate analogs. Key: (○) 5-fluoro orotate; (△) 5-methyl orotate; (□) 5-amino orotate; and (●) 5-bromo orotate. The control culture of *P. falciparum*-infected cells had radioactivity of 13,390 ± 960 cpm, whereas control red cells without parasites had 330 ± 40 cpm.

In vivo *antimalarial activity of orotate analogs.* Mice infected with *P. berghei* were treated daily for 4 days with three doses of 5-fluoro orotate, 5-amino orotate, 5-methyl orotate and orotate, and compared to chloroquine therapy. At a dose of 25 mg/kg, 5-fluoro orotate, 5-amino orotate and chloroquine cleared parasitemia in mice infected with *P. berghei*, 5-methyl orotate inhibited parasitemia by as much as 70%, whereas orotate itself had no inhibitory effect on parasitemia. The control mice who did not receive the inhibitors had parasitemia of 4.51 ± 1.06% and died 9 days postinfection. Mice treated with 5-fluoro orotate at a dose of 10 mg/kg showed no parasitemia. At a lower dose of 2.5 mg/kg, only 5-fluoro orotate had potent antimalarial activity against *P. berghei* in mice (93% inhibition of parasitemia).

Cytotoxicity of orotate analogs to mammalian cells. Since the purified enzymes DHOase and DHODase were obtained from *P. berghei*, a rodent malaria parasite, the toxicity of the orotate analogs was tested against mouse peritoneal macrophages. At the end of a 48-hr treatment, the cellular LDH activity of the macrophages treated with individual orotate analogs (76.4 ± 12.8 μmol/min/mg protein) at a concentration as high as 100 μM was the same as the orotate analog-free control macrophages (80.5 ± 11.5 μmol/min/mg protein). There was no microscopic evidence of increased cytoplasmic vacuolation or loss of cellular adherence to the culture surface of the treated macrophages.

DISCUSSION

These results indicate that inhibitors of DHOase and DHODase, important enzymes of the pyrimidine biosynthetic pathway, are active as antimalarial compounds against the erythrocytic stage of *P. falciparum in vitro*. These results are consistent with previous observations on some 5-substituted orotate analogs having antimalarial activity both *in vitro* against *P. falciparum* [10–12] and *P. knowlesi* [30] and *in vivo* against *P. yoelii* in mice [30].

A systematic study of the inhibition of DHOase by 5-substituted orotate analogs showed that the order of inhibition of the malarial *P. berghei* DHOase by these analogs did not follow the order of electronegativity of the substituent, but followed the size of the 5 substituent in position 5 of orotate. A marked increase in K_i values was found when the size of the substituent exceeded 3.5 Å, i.e. methyl < bromo < iodo, for the ring cyclization and cleavage reactions (Table 1). These results suggest some steric hindrance, but not hydrophobicity due to electronegativity, to binding with bulky substituents which may be located adjacent to the binding site of the 5-substituent. Similar findings were reported by Christopherson and Jones for mouse DHOase [26].

The orotate analogs that were the most effective inhibitors of the malarial DHOase activity were also the best inhibitors of DHODase activity, i.e. 5-fluoro, 5-amino and 5-methyl orotate (Table 2). 5-Bromo and 5-iodo orotate were the least effective inhibitors on both malarial DHOase and DHODase enzymes. These results for the 5-substituted orotate analogs are not generally consistent with previous observations on the rat enzyme in which the halogen substituents (electron-withdrawing) are better inhibitors than methyl and amino substituents (electron-donating) in position 5 of orotate [29].

The simple competitive inhibition patterns of orotate and some 5-substituted orotate analogs on both DHOase and DHODase from *P. berghei* were quite similar to those of the mammalian enzymes [26, 29]. However, 5-fluoro orotate was reported recently to interact with hamster DHOase in a noncompetitive inhibitory manner [31], which indicates a difference for the mammalian and malarial enzymes. This may be due to the marked difference of physical properties in which the

mammalian DHOase is a part of the M_r 240,000 multifunctional protein [7, 8], whereas the malarial enzyme exists as a monomeric protein [4].

Of the six compounds (orotate and its analogs) tested against *P. falciparum in vitro*. 5-fluoro orotate showed the greatest antimalarial activity (IC_{50} and IC_{90} of 10 and 60 nM, respectively), 5-methyl and 5-amino orotates had potent antimalarial activity (IC_{50}, 1 μM or more), whereas orotate, 5-bromo and 5-iodo orotates showed little effect, even at a concentration of 1 mM. Because orotate is an intermediate of the pyrimidine *de novo* synthetic pathway, it should not have any inhibition on the enzymes *in vivo*. The potent inhibitors have also shown antimalarial activity when tested against *P. berghei in vivo*. 5-Fluoro orotate had the greatest schizontocidal effect in mice infected with *P. berghei*. These *in vivo* effects are relevant to the effects of the inhibitors on *in vitro* growth of *P. falciparum* and purified *P. berghei* DHOase and DHODase. The cytotoxicity test suggests that these analogs have no effect on the mammalian cells tested. However, they show a cytotoxic effect on some mammalian cell lines [11, 13, 32].

The potent antimalarial activity of 5-fluoro orotate on *P. falciparum* growth *in vitro* and *P. berghei in vivo* may not only be due to its inhibition of the malarial DHOase and DHODase enzymes but may also involve an effect on other enzymes of pyrimidine *de novo* synthesis, i.e. OPRTase [33] and thymidylate synthase [32]. However, the inhibition by 5-fluoro orotate and its metabolite [32] of these malarial enzymes should be explored further. More recently, many transition-state analogs of DHOase have been synthesized and found to have no significant inhibition on the growth of several tumor cell lines [31, 34, 35]. Effective analogs are needed for further investigation.

Because of their inability to salvage preformed pyrimidines, malarial parasites must have their own pyrimidine biosynthesis [2–6]. The enzymes in the pyrimidine *de novo* synthetic pathway may represent potential targets for attack by several inhibitors, i.e. 5-substituted orotate analogs. The effects of the more potent compounds tested in combination with inhibitors of other enzymes, i.e. CPS II and ATCase, of the pathway on *P. falciparum in vitro* are currently under investigation. As a rational approach towards designing better inhibitors, especially those inhibitors which adversely affect pyrimidine *de novo* synthesis, promising antimalarial agents will soon be developed.

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