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Selective Activity of 5-Fluoroorotic Acid against *Plasmodium falciparum* In Vitro†

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Unlike mammalian cells, malarial parasites are completely dependent on de novo pyrimidine metabolism. Even though these parasites do not use external uracil or uridine, orotic acid, an intermediate of pyrimidine biosynthesis, is successfully transported into the parasite and incorporated into parasite nucleic acids. On this basis, it was hypothesized that 5-fluoroorotate, a cytotoxic derivative of orotic acid, may be a potent and selective antimalarial agent. In vitro, 5-fluoroorotate caused 50% inhibition of the growth of *Plasmodium falciparum* at a concentration of 6.0 nM. In contrast, 5-fluorouracil, 5-fluorouridine, and 5-fluoro 2′-deoxyuridine were much less effective against malarial parasites. Chloroquine-susceptible and chloroquine-resistant clones of *P. falciparum* were equally susceptible to 5-fluoroorotate. The toxicity of 5-fluoroorotate was evaluated on four human cell lines (HT-1080, IMR-90, HeLa S3, and HL-60) and one mouse cell line (L-1210). Compared with malarial parasites, the mammalian cells were relatively tolerant of 5-fluoroorotic acid (50% inhibitory concentration, 0.9 to 10 μM). Finally, in the presence of 1 mM uridine, all mammalian cells were partially protected from 5-fluoroorotate cytotoxicity, but uridine offered no protection to *P. falciparum*.

According to World Health Organization estimates of 1982, more than 350 million people live in areas of the world where malaria is highly endemic and where no special antimalarial measures are being applied (43). About 2.2 billion people live in areas where malaria is still endemic but where control measures have decreased the level of endemicity. *Plasmodium falciparum* strains resistant to chloroquine are confirmed in at least 40 tropical and subtropical countries (27). The future of malarial chemotherapy is particularly alarming in view of parasite strains that display cross-resistance to several structurally unrelated drugs (42; T. E. Hubbert, A. M. Oduola, D. L. Klayman, and W. K. Milhous, paper presented at the 35th Annu. Meet. Am. Soc. Trop. Med. Hyg. 1986). For these reasons, it is important to develop novel antimalarial drugs, particularly those effective against strains of *P. falciparum* that display multiple drug resistance.

The erythrocytic phase of the life cycle of *P. falciparum* is associated with the clinical symptoms of malaria. During this 48-h asexual cycle, each parasite inside an erythrocyte generates 6 to 24 offspring that burst out and individually invade fresh erythrocytes. The exponential increase in parasite DNA and RNA requires a steady supply of purine and pyrimidine nucleotides. Malarial parasites use the rich pool of adenine nucleotides inside the erythrocyte to obtain their supply of purines, but the parasites have to synthesize pyrimidines de novo (35).

Two lines of evidence suggested that malarial parasites were unable to utilize exogenous preformed pyrimidines. First, early studies showed that radioactive carbon dioxide was incorporated into pyrimidine units of malarial nucleic acids while uracil, uridine, thymine, thymidine, cytidine, and deoxycytidine were not (3, 13, 35, 39, 40). Later, several species of *Plasmodia* were shown to carry enzymes for de novo pyrimidine biosynthesis but not for the salvage of preformed pyrimidines (10–12, 17, 19, 30, 32). In contrast, mammalian cells utilize preformed pyrimidine bases and nucleosides by salvage pathways (18, 24, 41).

Any compound with schizonticidal activity has to be transported across the three membranes that separate the host plasma from the cytoplasm of the parasite. Gutteridge and Trigg demonstrated that radioactive orotic acid, an intermediate in de novo pyrimidine biosynthesis, was effectively transported and incorporated into *Plasmodium* nucleic acids (13). This led us to the hypothesis that an analog of orotic acid which inhibits de novo pyrimidine metabolism would inhibit the growth of malarial parasites.

To test this hypothesis, we considered 5-fluoroorotate as a model antimalarial drug. The uptake and metabolism of 5-fluoro-pyrimidines have not been studied in malarial parasites. Nevertheless, we expected the fluorinated analog to be transported into the parasite as effectively as orotic acid because the substitution of a fluorine for a proton at the 5 position represents a minimum structural alteration. In mammalian cells, 5-fluoroorotate is metabolized to toxic 5-fluoropyrimidines with the aid of orotate phosphoribosyltransferase, orotidylate decarboxylase, and other enzymes of de novo pyrimidine metabolism (7, 16, 31). Since malarial parasites express enzymes of de novo pyrimidine biosynthesis, we inferred that *P. falciparum*, too, would have the capacity to activate 5-fluoroorotate to toxic metabolites.

In this study, we examine the in vitro antimalarial properties of 5-fluoroorotate against the human pathogen *P. falciparum* in culture. We also report the effect of this compound on the proliferation of mammalian cells in culture. The data show that 5-fluoroorotate is selectively toxic to malarial parasites, particularly when it is used in combination with uridine. We also show that a chloroquine-resistant clone of *P. falciparum* is as vulnerable to 5-fluoroorotate as a chloroquine-susceptible clone.

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† P.K.R. dedicates this paper to the memory of Shree Kesarji K. Rathod.

MATERIALS AND METHODS

5-Fluoropyrimidines. 5-Fluoroorotate (ZN-80849) was obtained from Walter Reed Army Institute of Research. All other pyrimidine compounds were purchased from Sigma Chemical Co., St. Louis, Mo.

Parasites. The development of two clones of human malaria parasites has been described previously (26). Clone D6 was from a West African isolate. Clone W2 was from a mixed culture; however, it resembled an Indochina isolate (26). The parasites were maintained by using standard in vitro techniques (15, 37).

Mammalian cells. Human HT-1080 cells were obtained from J. Greene, The Catholic University of America, Washington, D.C. (29). R. Nardone, The Catholic University of America, provided us with the following cell lines: human IMR-90 (25), human HeLa S3 (28), human HL-60 (6), and mouse L-1210 (23).

HT-1080 cells were maintained in Eagle minimum essential medium (EMEM) supplemented with 5% fetal bovine serum and 2 mM glutamine. Medium for the growth of IMR-90 cells was prepared from autoclavable EMEM (Flow Laboratories, Inc., McLean, Va.) supplemented with 10% fetal bovine serum, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 0.21% sodium bicarbonate (wt/vol), and 2 mM L-glutamine. For the HeLa S3 cells, autoclavable EMEM was supplemented with 5% donor calf serum, 20 mM HEPES, 0.21% sodium bicarbonate (wt/vol), and 2 mM glutamine. The HL-60 cells were grown in autoclavable EMEM supplemented with 10% fetal bovine serum, 15 mM HEPES, 0.21% sodium bicarbonate, 2 mM L-glutamine, and 1 mM sodium pyruvate. Finally, the L-1210 cells were maintained in Dulbecco medium as described by Stern et al. (36).

Antimalarial activity. The following procedure was used for routine assay of antimalarial activity. Various concentrations of fluoropyrimidines, dispensed in 25 μl of culture medium, were added to individual wells of a 96-well microdilution plate (8). Erythrocytes with 0.25 to 0.5% parasitemia were added to each well in 200 μl of culture medium to give a final hematocrit of 1.5%. The plates were incubated at 37°C for 48 h under a 5% CO_2–5% O_2 atmosphere. Finally, the uptake of 0.5 μCi of [G-^{3}H]hypoxanthine (1 Ci/mmol) in each well was examined as described previously (8).

Among malarial parasitologists, the uptake of [^{3}H]hypoxanthine has become an established method for measuring parasite viability and proliferation (8). However, for one specific study, the antimalarial activity of 5-fluoroorotate was measured by three independent methods. Three sets of infected erythrocytes were incubated for 48 h with 5-fluoroorotate. Each set contained erythrocytes exposed to different levels of 5-fluoroorotate. The incubation conditions were as described above, with one exception: isoleucine concentration in the growth medium was lowered to 20% of normal values. At the end of drug treatment, individual wells in one set were treated with [^{3}H]hypoxanthine (0.5 μCi; 1 Ci/mmol), in another set they were treated with [U-^{14}C] isoleucine (0.5 μCi; 0.33 Ci/mmol), and in the third set they were treated with medium alone. After 18 h, we determined the amount of radioactivity incorporated into erythrocyte macromolecules in the first and second sets of erythrocytes (8). Samples from the third set of infected erythrocytes were smeared on microscope slides and treated with Giemsa stain, and the ratio of infected to noninfected erythrocytes was determined.

Toxicity to human cells. Mammalian cells were seeded into six-well culture plates at a density of 50,000 cells per 9.6-cm^2 well. Each well contained 2.5 ml of growth medium. The following day, fresh culture medium containing the appropriate fluoropyrimidine was added to the wells and the plates were incubated at 37°C for 48 h in a 5% CO_2 incubator. Then the number of cells in each well was determined.

The HT-1080, IMR-90, and HeLa S3 cells attach to culture plates. They were first washed with calcium- and magnesium-free phosphate-buffered saline (PBS-CMF; 136 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4) and then treated with 1 ml of 0.05% trypsin–0.02% EDTA in PBS-CMF. After 1 min, the detached cells were suspended in 1 ml of EMEM and collected by centrifugation at 300 × *g* for 3 min. The cell pellets were suspended in 1 ml of PBS-CMF. Half of this suspension was mixed with 0.4 ml of PBS-CMF and 0.1 ml of trypan blue solution (0.5% trypan blue dye in 0.85% saline), and the concentrations of viable cells were determined with a hemacytometer.

The HL-60 and L-1210 cells grew in suspension. Therefore, it was unnecessary to treat these cells with trypsin at the end of the 48-h incubation. Cells from this group were transferred directly to microcentrifuge tubes and pelleted by centrifugation at 300 × *g* for 3 min. The pellets were suspended in 0.9 ml of PBS-CMF, mixed with 0.1 ml of trypan blue solution, and counted with the aid of a hemacytometer.

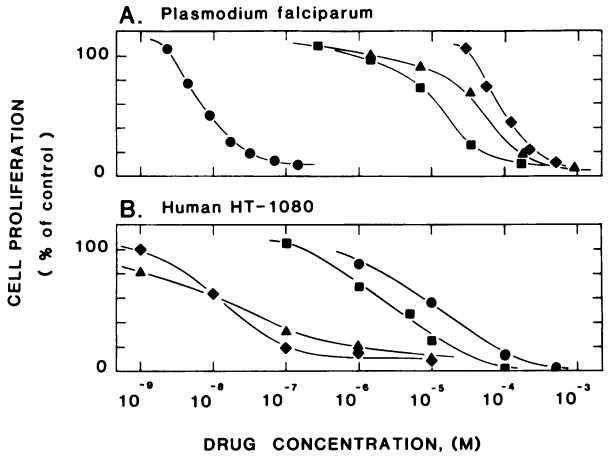

FIG. 1. Inhibition of proliferation of cultured *P. falciparum* (A) and human HT-1080 (B) cells by fluoropyrimidines. In the antimalarial experiment, incorporation of radioactivity from tritiated hypoxanthine into nucleic acids served as a measure of parasite proliferation (see Materials and Methods). Control wells incorporated 11,200 cpm of tritium from labeled hypoxanthine into precipitable material. Each datum point represents an average of two determinations. In the study of toxicity to human cells, proliferation of cells was determined directly by measuring the number of viable cells per well (see Materials and Methods). Control wells showed a net increase of 350,000 cells during the 48-h incubation. Each datum point represents an average of two determinations. Symbols: ●, 5-Fluoroorotate; ■, 5-fluorouracil; ▲, 5-fluorouridine; ◆, 5-fluoro 2'-deoxyuridine.

RESULTS

Antimalarial activity of 5-fluoropyrimidines. In an in vitro test, 5-fluoroorotate showed potent antimalarial activity against an Indochina clone of *P. falciparum* (50% inhibitory concentration [IC_{50}], 6.0 nM; Fig. 1A). 5-Fluorouracil, 5-

TABLE 1. Comparison of inhibitory activities of 5-fluoroorotate and standard antimalarial drugs against *P. falciparum* clones

Drug	Indochina clone		Sierra Leone clone	
	IC_{50} (nM)[a]	Molar index[b]	IC_{50} (nM)[a]	Molar index
5-Fluoroorotate	6.0 ± 1.7	1	5.8 ± 3.1	1.0
Chloroquine	127 ± 23	21	9.0 ± 1.7	1.6
Quinine	83 ± 5	14	12.7 ± 6.8	2.2
Pyrimethamine	111 ± 29	19	0.24 ± 0.16	0.04
Sulfadoxine	25,593 ± 7,077	4,344	76.2 ± 13.2	13.5

[a] Means ± standard deviations of five independent drug response curves.
[b] Molar indices illustrate the in vitro equimolar activities of 5-fluoroorotate relative to established drugs. For example, 5-fluoroorotate is 21 times more active than chloroquine against the multidrug-resistant Indochina clone and 1.6 times more active than chloroquine against the chloroquine-susceptible Sierra Leone clone.

fluorouridine, and 5-fluoro 2'-deoxyuridine were much less effective as antimalarial agents; these compounds showed IC_{50}s of 12, 32, and 100 μM, respectively (Fig. 1A).

The in vitro antimalarial activity of 5-fluoroorotate was measured side-by-side with established antimalarial drugs. Two different clones of *P. falciparum* were used for this study, a chloroquine-resistant Indochina clone and a chloroquine-susceptible clone from West Africa. Unlike the other antimalarial agents tested, 5-fluoroorotate was equally effective against both clones of malarial parasites (Table 1).

In the studies described so far, uptake of radioactive hypoxanthine served as a marker of cell proliferation. Since this investigation deals with the antimalarial effects of a pyrimidine analog, it was important to determine whether the uptake of hypoxanthine (a purine) was a valid marker of parasite proliferation. The toxicity of 5-fluoroorotate against *P. falciparum* was tested by three different assay methods. With the West African clone of *P. falciparum*, 5-fluoroorotate showed an IC_{50} of 2.2 nM when hypoxanthine uptake was used as an indicator of parasite proliferation, an IC_{50} of 4.7 nM when isoleucine uptake served as such a measure, and an IC_{50} of 2.5 nM when the increase in parasitemia was determined directly with a light microscope. These results reassured us that a decrease in hypoxanthine uptake was indeed a valid measure of 5-fluoroorotate toxicity to malarial parasites in culture.

Potential toxicity of 5-fluoroorotate to mammalian cells. The ability of 5-fluoroorotate to inhibit the proliferation of mammalian cells was first studied with human fibrosarcoma cells (Fig. 1B). It required 10 μM 5-fluoroorotate to cause 50% inhibition of these human cells as compared with 6 nM to achieve the same results with *P. falciparum*. This mammalian cell line was less vulnerable to 5-fluoroorotate than to the commonly used anticancer drugs 5-fluorouracil, 5-fluorouridine, and 5-fluoro 2'-deoxyuridine (IC_{50}s of 2 μM, 30 nM, and 10 nM, respectively; Fig. 1B).

To determine whether mammalian cells, in general, were less susceptible to 5-fluoroorotate, the toxicity of this compound was examined against a battery of four human cell lines and one rapidly dividing mouse cell line (Table 2). Even though the IC_{50}s varied between 1 and 10 μM, none of the mammalian cell lines approached the vulnerability of malarial parasites.

Selective rescue of mammalian cells from 5-fluoroorotate toxicity. Since mammalian cells transport and utilize pyrimidine nucleosides efficiently and malarial cells do not, inclusion of uridine with 5-fluoroorotate was expected to protect mammalian cells selectively from cytotoxicity by saturating

TABLE 2. Comparison of inhibitory activities of 5-fluoroorotate against different mammalian cell lines

Cell line	Cell origin	IC_{50} (μM)	
		Without uridine	With 1 mM uridine
HT-1080	Human fibrosarcoma	10	50
IMR-90	Human lung fibroblast	1.1	2.2
HeLa S3	Human cervix epithelioid carcinoma	2.0	3.5
HL-60	Human promyelocytic leukemia	0.9	4.5
L-1210	Mouse lymphocytic leukemia	2.8	5.0

the pool of pyrimidine nucleotides with nonfluorinated metabolites. The cytotoxicity of 5-fluoroorotate to human HT-1080 cells was decreased fivefold by simultaneous administration of 1 mM uridine (Fig. 2). All five mammalian cell lines tested were partially protected from 5-fluoroorotate when 1 mM uridine was included in the toxicity assay medium (Table 2). Depending on the cell line, the IC_{50}s of 5-fluoroorotate were two to five times higher in the presence of 1 mM uridine.

In contrast, uridine had no effect on the cytotoxicity of 5-fluoroorotate towards malarial parasites (Fig. 2).

DISCUSSION

The differences in pyrimidine metabolism between mammalian cells and malarial parasites have generated interest in developing selective antimalarial agents targeted at de novo pyrimidine biosynthesis. Scott et al. showed that pyrazofurin, an inhibitor of orotidylate decarboxylase, blocks in vitro proliferation of *P. falciparum* with an IC_{50} of 6 to 10 μM (34). Hammond et al. showed that 2-(4-*t*-butylcyclohexyl)-3-hydroxy-1,4-naphthoquinone, an inhibitor of dihydroorotate dehydrogenase, inhibits the de novo pyrimidine pathway of *P. falciparum* at concentrations as low as 0.1 nM (14). In 1974, as part of a large drug-screening effort, McCormick et al. reported the effects of 62 analogs of purines and pyrimidines on nucleic acid metabolism in the simian malaria *P. knowlesi* (22). One of these compounds was 5-fluoroorotate. At the single concentration tested (60 μM), 5-fluoroorotate was shown to inhibit the incorporation of radioactive adenosine and orotic acid into DNA. The study did not determine the minimum quantity of drug necessary to inhibit the

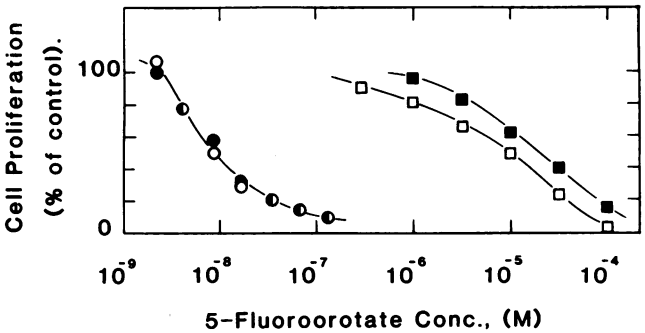

FIG. 2. Effects of uridine on cytotoxicity of 5-fluoroorotate. Cells were grown for 48 h with various concentrations of 5-fluoroorotate. Symbols: ○ and ●, Indochina clone W-2 of *P. falciparum* without uridine (○) and with 1 mM uridine (●); □ and ■, human HT-1080 cells without uridine (□) and with 1 mM uridine (■). Half-shaded symbols represent overlapping datum points.

proliferation of malarial parasites nor did it suggest how an inhibitor of de novo pyrimidine metabolism might be used to kill parasite cells selectively without harming mammalian cells.

In this report it is shown that the IC_{50} of 5-fluoroorotate against *P. falciparum* is 6 nM. The molecular basis for the remarkable potency of 5-fluoroorotate as an antimalarial agent is not known. The uptake of 5-fluoropyrimidines into infected erythrocytes and their subsequent conversion to toxic metabolites remain to be studied.

Based on what is known about the metabolism of nonfluorinated pyrimidines in malarial parasites, it is likely that two factors contribute to the potent antimalarial activity of 5-fluoroorotate. First, the same conditions that allow malarial parasites to transport and activate radioactive orotic acid for nucleic acid synthesis probably participate in converting exogenous 5-fluoroorotate to toxic 5-fluoropyrimidine nucleotides in the parasite cytoplasm. These conditions may include the ability to transport the drug into the parasite and its activation through orotate phosphoribosyltransferase and orotidylate decarboxylase (31). Second, since parasites are completely dependent on de novo pyrimidine biosynthesis, the possibilities for diluting the toxic fluoropyrimidines by nonfluorinated nucleotides must be limited. Low levels of endogenous 5-fluoro metabolites may be enough to cause potent toxicity. This line of thinking is supported by our observations on the antimalarial activity of 5-fluoropyrimidines other than 5-fluoroorotate. Even though exogenous radioactive uracil and uridine are not effectively incorporated into parasite nucleic acids (3, 13, 35, 39, 40), the corresponding 5-fluoropyrimidines have some antimalarial activity (Fig. 1A). The low but significant antimalarial activity of 5-fluorouracil could arise from limited activation of this drug by orotate phosphoribosyltransferase (31, 38). The weak antimalarial activities of 5-fluorouridine and 5-fluoro 2′-deoxyuridine are more difficult to explain. Malarial parasites do not have detectable levels of pyrimidine nucleoside kinases (32). It is possible that the fluorinated nucleosides are first degraded to 5-fluorouracil before they cause cytotoxicity.

A major mechanism of 5-fluoroorotate toxicity may involve its intracellular conversion to 5-fluoro 2′-deoxyuridylate, which is a potent inactivator of thymidylate synthetase (20, 33). Unlike the mammalian enzyme, protozoan thymidylate synthetase exists as part of a bifunctional protein that also includes dihydrofolate reductase activity (4, 5, 9). It remains to be seen whether this important difference in protein structure plays a role in the selective antimalarial activity of 5-fluoroorotate toxicity.

In addition to the potent in vitro antimalarial activity of 5-fluoroorotate, three observations encourage us to consider the potential therapeutic significance of our results. One, the activities of this compound against chloroquine-susceptible and chloroquine-resistant *P. falciparum* are identical; this suggests that 5-fluoroorotate ought to be considered a potential antimalarial agent against multiple-drug-resistant organisms. Two, 5-fluoroorotate has limited activity against a variety of mammalian cell lines. This may be due to the poor transport of 5-fluoroorotate and orotate into mammalian cells (1, 2). Three, uridine selectively decreases 5-fluoroorotate toxicity towards mammalian cells but not parasite cells. It is known that the toxicity of 5-fluorouracil to mice can be reversed with uridine, even when the rescuing agent is delivered significantly after the fluoropyrimidine (21). Since 5-fluoroorotate is less toxic to mammalian cells than 5-fluorouracil, it is possible that treatment of the host animal with uridine before and after 5-fluoroorotate administration could make the latter drug tolerable at doses that have potent antimalarial activity.

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