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Antimalarial Agents Directed at Thymidylate Synthase

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After many years of decline, malaria is re-emerging as a major health threat around the world (Oaks et al 1991). New treatments against *Plasmodium falciparum* are necessary because parasites are rapidly developing resistance to existing drugs (White 1996).

Many biochemical studies on protozoan parasites accentuate enzymes and metabolic pathways that are potential targets for selective chemotherapy. However, most clinically useful drugs still arise through empirical screening of natural products or synthetic analogues of previously successful antimalarial agents. The scarcity of new antimetabolites against malarial parasites is a measure of our limited ability to exploit differences in metabolic pathways between mammalian cells and malarial parasites for selective chemotherapy.

Recently, it has been possible to identify potent and selective antimalarial agents by directing antimetabolites at malarial thymidylate synthase.

Rationale for Targeting Thymidylate Synthase

Pyrimidine metabolism in malaria

The erythrocytic phase of the life cycle of *P. falciparum* is associated with the clinical symptoms of malaria. During this 48 h cycle, each parasite inside a red blood cell generates about 24 offspring that burst out and reinvade new host cells. The exponential increase in parasite DNA and RNA requires a proportional supply of purine and pyrimidine nucleotides. Malarial parasites use the rich pool of adenine nucleotides in erythrocytes to obtain purines, but the parasites have to synthesize pyrimidines de novo (Gutteridge & Trigg 1970; Sherman 1979; Reyes et al 1982).

Erythrocytic stages of malarial parasites are completely dependent on the de novo pyrimidine pathway. Malarial parasites fail to incorporate exogenous radiolabelled pyrimidine bases and pyrimidine nucleosides into nucleic acids (Gutteridge & Trigg 1970; Sherman 1979). Additionally, erythrocytic stages of *P. falciparum* are rich in enzymes for de novo pyrimidine biosynthesis but lack the enzymes for salvage of pyrimidine bases or nucleosides (Reyes et al 1982). Orotic acid, an intermediate in de novo pyrimidine biosynthesis, can be metabolized by malarial parasites (Gutteridge & Trigg 1970; Reyes et al 1982; Rathod & Reyes 1983).

In contrast to malarial parasites, most mammalian cells have both the capacity for de novo synthesis of pyrimidine nucleosides as well as salvage of preformed pyrimidines (Jones 1980; Moyer et al 1985). On this basis, one would suspect that a potent, selective inhibitor of any of the steps in de novo pyrimidine biosynthesis would be a selective inhibitor of malarial parasite proliferation, particularly when used in combination with nucleosides. Host-parasite differences in the structure of the target enzymes would be of additional benefit but would not be entirely necessary.

Biosynthesis of thymidylate in malaria

Thymidylate synthase plays an important role in the biosynthesis of DNA precursors. Using methylenetetrahydrofolate as a co-substrate, this enzyme converts 2′-deoxyuridylate to thymidylate (Carreras & Santi 1995). Continued activity of thymidylate synthase is dependent on an active dihydrofolate reductase which regenerates tetrahydrofolate from dihydrofolate (Schweitzer et al 1990).

Dihydrofolate reductase activity and thymidylate synthase activity reside on a single polypeptide encoded by a single gene in all protozoan parasites examined (Garrett et al 1984). Malarial dihydrofolate reductase-thymidylate synthase has been cloned and sequenced (Bzig et al 1987). The bifunctional enzyme has been purified from malarial parasites in culture and from heterologous expression systems (Zolg et al 1989; Sirawaraporn et al 1990; Hekmat-Nejad & Rathod 1996).

Of all the steps in de novo pyrimidine metabolism, the biosynthesis of thymidylate appears to be a particularly attractive target for chemotherapy because, in many cell types, even partial inhibition of this step is known to result in nucleotide imbalances and cell death (Ingraham et al 1986; Yoshioka et al 1987; Houghton et al 1989; Kunz et al 1994). Selective inhibition of malarial dihydrofolate reductase by compounds such as pyrimethamine and cycloguanil is responsible for the antimalarial activity of pyrimethamine and proguanil (Carrington et al 1951; Hitchings 1960; Ferone et al 1969).

Resistance to these antimalarial agents is most frequently associated with point mutations in the dihydrofolate reductase domain of the bifunctional enzyme (Foote et al 1990; Peterson et al 1990). Pyrimethamine-resistance can be acquired via transfection of a gene encoding a pyrimethamine-resistant dihydrofolate reductase-thymidylate synthase (Donald & Roos 1993; Crabb & Cowman 1996; van Dijk et al 1996; Wu et al 1996).

Until recently, strategies for selective inhibition of malarial thymidylate synthase domain were nonexistent. The problem appeared particularly challenging because thymidylate synthase is considered to be among the most conserved protein in nature (Carreras & Santi 1995). In terms of steady-state kinetics, there are no differences between malarial and mammalian thymidylate synthase (Table 1).

5-Fluoro-2′-deoxyuridylate and thymidylate synthase

A 5-fluorinated analog of 2′-deoxyuridylate is a potent inactivator of thymidylate synthase from mammalian cells and other sources (Reyes & Heidelberger 1965; Heidelberger et al 1983; Carreras & Santi 1995). The normal mechanism of thymidylate synthase includes a tertiary complex involving covalent attachments between the enzyme thymidylate synthase, 2′-deoxyuridylate, and the co-substrate methylenetetrahydrofolate (Santi 1981). Subsequent steps in the catalytic

Table 1. A comparison of the kinetic constants of thymidylate synthase from *P. falciparum* and from mammalian cells.

Kinetic property	*P. falciparum*		Mammalian enzyme
	Recombinant enzyme	Native enzyme	
K_m, 2′-deoxyuridylate	2·0 μM	1·3 μM	1·8 μM, 3·4 μM
K_m, methylenetetrahydrofolate	39 μM	30 μM	31 μM, 8 μM, 27 μM
k_{cat}, (per FdUMP binding site)	118 min^{-1}	120 min^{-1}	150 min^{-1}
K_i, FdUMP	2·0 nM	–	1·7 nM, 5 nM
K_i, D1694 monoglutamate	2·80 μM	–	0·06 μM
K_i, D1694 pentaglutamate	1·5 nM	–	1·0 nM

Hekmat-Nejad & Rathod (1996).

reaction require extraction of a proton from the 5-position of the pyrimidine ring. In the presence of methylenetetrahydrofolate and 5-fluoro-2′-deoxyuridylate, thymidylate synthase forms an analogous ternary species but this complex is stable and fails to dissociate in the forward direction due to the fluorine in the 5-position of the pyrimidine ring.

The K_i of 5-fluoro-2′-deoxyuridylate for malarial thymidylate synthase is about 2 nM when tested in the presence of 100 μM methylenetetrahydrofolate (Table 1; Hekmat-Nejad & Rathod 1996). This value is comparable with the potency of 5-fluoro-2′-deoxyuridylate against thymidylate synthase molecules from human and other mammalian sources (Reyes & Heidelberger 1965; Dolnick & Cheng 1977; Davisson et al 1989).

Antimalarial Activity of 5-Fluoroorotate In-Vitro

Generating 5-fluoro-2′-deoxyuridylate in malarial parasites

While 5-fluoro-2′-deoxyuridylate is a potent inhibitor of thymidylate synthase, this molecule itself is not well suited for inhibiting proliferation of cells, since most cells are not permeable to nucleotides. Compounds such as 5-fluorouracil, 5-fluorouridine, and 5-fluoro-2′-deoxyuridine, which can act as metabolic precursors of 5-fluoro-2′-deoxyuridylate, are potent inhibitors of some proliferating mammalian cells. Since malarial parasites lack enzymes for salvage of standard pyrimidines such precursors were not expected to have potent antimalarial activity. Indeed, 5-fluorouracil, 5-fluorouridine, and 5-fluoro-2′-deoxyuridine are far less effective against malarial parasites than against mammalian cells in culture (Rathod et al 1989).

Based on precursor incorporation studies and enzyme profiles in *P. falciparum*, it was known that exogenous orotic acid, an intermediate in de novo pyrimidine biosynthesis, was utilized by malarial parasites. It was also known that, at least in other cells, enzymes responsible for orotic acid metabolism could convert 5-fluoroorotic acid into toxic fluorinated nucleotides. It was hypothesized that 5-fluoroorotic acid would be a potent antimalarial agent and this potency would be closely related to inactivation of malarial thymidylate synthase activity.

In-vitro activity of 5-fluoroorotate against P. falciparum

5-Fluoroorotate is a potent and selective antimalarial agent (Rathod et al 1989; Gomez & Rathod 1990). The IC50 of 5-fluoroorotate against *P. falciparum* in culture was about 6 nM (Table 2). About 0·1 μM 5-fluoroorotate was sufficient to completely inhibit proliferation of malarial parasites in culture. Malarial parasites resistant to chloroquine, quinine, pyrimethamine, and sulphadoxine were as susceptible to 5-fluoroorotate as traditional drug-sensitive parasites (Table 2). Addition of preformed pyrimidines such as uridine to the culture medium did not alter the susceptibility of malarial parasites to 5-fluoroorotate, consistent with predictions based on precursor incorporation studies and assay of enzyme activities in malarial parasites (Reyes et al 1982).

Inactivation of malarial thymidylate synthase

Uninfected erythrocytes lack detectable thymidylate synthase, so the status of thymidylate synthase in 5-fluoroorotate treated malarial parasites could be determined by directly assaying lysates of infected erythrocytes for the ability to release tritiated water from 5-[^{3}H] deoxyuridine monophosphate (Rathod et al 1992). The same doses of 5-fluoroorotate that were sufficient to inhibit proliferation of malarial parasites were also sufficient to inactivate thymidylate synthase activity in infected erythrocytes (Rathod et al 1992). Control studies demonstrated that during the early phases of thymidylate synthase inactivation, dihydrofolate reductase on the other half of the bifunctional enzyme remained active (Rathod et al 1992). This was consistent with the hypothesis that loss of thymidylate synthase activity in 5-fluoroorotate-treated cells was specific and not due to general necrosis.

Thymidylate synthase inactivation was expected to lead to nucleotide imbalances and cell death (Ingraham et al 1986; Yoshioka et al 1987; Houghton et al 1989; Kunz et al 1994). Indeed, a clonal viability assay for *P. falciparum* was developed and it confirmed that the doses of 5-fluoroorotate that were sufficient to inhibit thymidylate synthase activity and parasite proliferation were also sufficient to kill parasites (Young & Rathod 1993). Similarly, there was a close relationship between the duration of exposure to 5-fluoroorotate necessary to inhibit thymidylate synthase and loss of cell viability.

An alternate mechanism for toxicity from 5-fluoropyrimidines involves incorporation of fluorinated nucleotides into RNA molecules (Evans et al 1980). Usually this mode of toxicity is associated with substitution of at least 2% of uridine molecules in RNA with 5-fluorouridine molecules. In malarial parasites, nanomolar concentrations of 5-fluoroorotate are not

Table 2. Antiproliferative activity of 5-fluoroorotate against *P. falciparum* and against human HT-1080 cells.

Antimalarial agent	*P. falciparum* cells		Human cells Fibrosarcoma HT-1080
	Drug-sensitive clone D6 from Sierra Leonne	Drug-resistant clone W2 from Indochina	
5-Fluoroorotate	6 nM	6 nM	10 000 nM
5-Fluoroorotate plus 1 mM uridine	6 nM	6 nM	50 000 nM

Values represent 50% inhibitory concentration. Rathod et al (1989).

sufficient to cause significant incorporation of 5-fluorouridine into parasite RNA or DNA (Rathod et al 1992).

Toxicity to mammalian cells

While the in-vitro potency of 5-fluoroorotate was encouraging, it was equally important to evaluate the potential toxicity of 5-fluoroorotate against mammalian cells in culture (Rathod et al 1989). It required higher than 1 μM 5-fluoroorotate before significant toxicity was observed against 5 different types of mammalian cells in culture. 5-Fluoroorotate was much less toxic to mammalian cells in culture than the commonly used anticancer agent 5-fluorouracil. Unlike malarial parasites in culture, all mammalian cells showed decreased susceptivity to 5-fluoroorotate in the presence of uridine (Table 2; Rathod et al 1989). This was consistent with biochemical observations that mammalian cells, unlike malarial parasites, usually express enzymes for the salvage of pyrimidine nucleosides.

In-vivo Efficacy

Pharmacokinetics of 5-fluoroorotate in mice

In-vitro studies had demonstrated that 0·1–1·0 μM 5-fluoroorotate was sufficient to inhibit proliferation of malarial parasites but not enough to cause significant toxicity to mammalian cells in culture, particularly in the presence of uridine. In an effort to reproduce these conditions in-vivo, mice were injected with varying doses of 5-fluoroorotate and the serum concentrations of 5-fluoroorotate were determined (Gomez & Rathod 1990). Such pharmacokinetic studies revealed that at least 1 mg kg^{-1} of 5-fluoroorotate had to be administered intraperitonneally to obtain 0·1–1·0 μM 5-fluoroorotate in serum. Since the half-life of 5-fluoroorotate was about 90 min, multiple injections of 5-fluoroorotate were anticipated for treating animals infected with malarial parasites.

Curing malaria in mice

Mice harbouring potentially lethal forms of *P. yoelii* at 5% parasitemia could be cured with 5-fluoroorotate without toxicity (Gomez & Rathod 1990; Rathod & Gomez 1991). Doses of 0·2–1·0 mg kg^{-1} six times a day for three days cured all the mice, but only after a significant recrudescence phase. Doses as high as 5 mg kg^{-1} were necessary to avoid recrudescence.

Serum levels necessary to avoid recrudescence

Pharmacokinetics studies had revealed that the doses of 5 mg kg^{-1} in mice produced serum levels of 1–10 μM. These concentrations of 5-fluoroorotate were much higher than those necessary to inhibit the proliferation of *P. falciparum* in culture. If recrudescence was due to resistant parasites, it appeared that 10 μM 5-fluoroorotate may be necessary not just to eliminate the majority of the parasites but to eliminate every single parasite, thereby avoiding resistance.

Uridine rescue to avoid toxicity

Repeated treatment with 5 mg kg^{-1} of 5-fluoroorotate, without uridine, was toxic to mice. In light of the pharmacokinetics, this was not surprising because 10 μM 5-fluoroorotate was toxic to most mammalian cells in the absence of uridine. However, the high doses of 5-fluoroorotate necessary to avoid recrudescence were readily tolerated when uridine was administered with 5-fluoroorotate (Gomez & Rathod 1990; Rathod & Gomez 1991). Mice treated with high doses of 5-fluoroorotate and uridine demonstrated no signs of weight loss, diarrhoea, or leucopenia. Furthermore, while reducing toxicity to the host animal, as seen with malarial parasites in culture, uridine did not compromise the antimalarial potency of 5-fluoroorotate.

Oral availability of 5-fluoroorotate

Mice inoculated with *P. yoelii* were cured by allowing them to drink 5-fluoroorotate freely for three days (Rathod & Gomez 1991). Since mice avoided uridine in the diet or in drinking water, this rescuing nucleoside had to be provided by intraperitoneal injections.

5-Fluoroorotate Resistance

Selection of resistant parasites in-vitro

Malarial parasites exposed to 0·1 μM 5-fluoroorotate generate clones that were 100–400 fold resistant (Rathod et al 1994). Biochemically, the mutant parasites used exogenous orotic acid about 40-fold less efficiently than parental strains. This suggested that the mutants either had diminished capacity to transport exogenous orotate and 5-fluoroorotate or had diminished capacity to activate 5-fluoroorotate to toxic pyrimidine nucleotides. Other explanations seemed less likely. The resistant parasites did not demonstrate increased utilization of exogenous nucleosides. The parasites were not cross-resistant to pyrimethamine, methotrexate, 5-fluorouracil, 5-fluorouridine, or 5-fluoro-2′-deoxyuridine. This suggested that amplification or alteration of dihydrofolate reductase-thymidylate synthase was unlikely to be responsible for 5-fluoroor-

otate resistance The resistance trait was stable in the absence of drug pressure and thus appeared to be of chromosomal origin.

Frequency of 5-fluoroorotate resistance

An in-vitro method was developed to estimate the frequency of resistance of *P. falciparum* to 5-fluoroorotate (Gassis & Rathod 1996). It was demonstrated that *P. falciparum* clone W2, which was already resistant to most antimalarial agents used in the field, developed resistance to 0·1 μM 5-fluoroorotate with a frequency of 10^{-6}. Recent data from this laboratory indicate that this high frequency may not be common to all clones of malarial parasites (Rathod et al unpublished).

Combinations of 5-fluoroorotate and atovaquone

In addition to 5-fluoroorotate, clone W2 of *P. falciparum* also readily developed resistance to atovaquone (Hudson 1993; Gassis & Rathod 1996). The frequency of resistance to 0·01 μM atovaquone was 10^{-5}.

Resistance to 5-fluoroorotate and atovaquone could be completely avoided by using a combination of 0·1 μM 5-fluoroorotate and 0·01 μM atovaquone (Gassis & Rathod 1996). There was no obvious synergy between 5-fluoroorotate and atovaquone; improved effectiveness was due to lack of cross-resistance between these compounds. Finally, a combination of 5-fluoroorotate and atovaquone did not increase toxicity to mammalian cells. Since these concentrations of antimalarial agents are easily tolerated by mammalian cells in culture, and easily tolerated in serum of mice, it is predicted that a combination of 5-fluoroorotate and atovaquone should eliminate malarial parasites in animals without recrudescence, without toxicity, and without the need to use uridine.

Folate-based Inhibitors of Thymidylate Synthase

New folate-based inhibitors of thymidylate synthase inhibit malarial thymidylate synthase at low nanomolar concentrations (Table 1; Hekmat-Nejad & Rathod 1996). These compounds have enormous potential as new antimalarial agents since their toxicity against mammalian cells, but not malarial cells, can be completely reversed with thymidine (Rathod & Reshmi 1994).

Conclusions

Basic studies on pyrimidine metabolism predicted that 5-fluoroorotate would be a potent and selective inhibitor of malaria proliferation and that this inhibition would be mediated by inactivation of thymidylate synthase. Careful preclinical studies are expected to further pave the way for the use of 5-fluoroorotate in field studies, particularly in combination with other antimalarial agents.

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