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Susceptibility of *Plasmodium falciparum* to a Combination of Thymidine and ICI D1694, a Quinazoline Antifolate Directed at Thymidylate Synthase

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Unlike mammalian cells, malarial parasites lack the enzymes to salvage preformed pyrimidines. For this reason, a combination of a thymidylate synthase inhibitor and the nucleoside thymidine should provide selective antimalarial activity even in the absence of any known active site differences between malarial and mammalian thymidylate synthases. To test this hypothesis, we evaluated the in vitro antimalarial activity of ICI D1694, a quinazoline antifolate that inhibits thymidylate synthase in mammalian cells. ICI D1694 inhibited the in vitro proliferation of *Plasmodium falciparum* with a 50% inhibitory concentration of 20 μM. As predicted, this antimalarial activity was not affected by the presence of 10 μM thymidine in the culture medium. In contrast, five different mammalian cells, several of which were susceptible to nanomolar levels of ICI D1694 in the absence of thymidine, were rescued by thymidine. At doses of 100 μM ICI D1694 and 10 μM thymidine, the proliferation of parasites was completely inhibited, but the proliferation of all mammalian cells remained unaffected. A test of susceptibility patterns among five different isolates of *P. falciparum* revealed that strains resistant to pyrimethamine, cycloguanil, or chloroquine had susceptibilities to ICI D1694 essentially the same as those of wild-type parasites. These findings are consistent with the hypothesis that, intracellularly, ICI D1694 inhibits *P. falciparum* thymidylate synthase. Overall, it is clear that even with an inhibitor of malarial thymidylate synthase that is not particularly effective in itself, one can obtain selective inhibition of parasites if the antimalarial agent is used in combination with thymidine. More effective inhibitors of malarial thymidylate synthase will undoubtedly lead to selective chemotherapy in vivo.

The emergence of *Plasmodium falciparum* cells that are resistant to traditional antimalarial agents, such as antifolates and chloroquine, has stimulated interest in new strategies for malaria chemotherapy. Over 40 years ago, efforts to screen synthetic analogs of folic acid for selective antimalarial activity led to the discovery of pyrimethamine and proguanil (1, 6, 9, 15, 28, 30). The molecular basis for the therapeutic activities of these compounds lays in the structural difference between malarial and mammalian dihydrofolate reductases: the parasite enzyme was 2,000-fold more susceptible than the mammalian counterpart to these types of compounds (10). While the ability of malarial parasites to develop resistance to these antifolates was recognized decades ago, only recently have we fully understood the molecular basis for drug resistance; *P. falciparum* develops resistance to pyrimethamine and cycloguanil by altering the structure of the enzyme dihydrofolate reductase (5, 8, 16, 20–22, 34). These modifications allow the enzyme to function as a catalyst but reduce the affinity of the active site for the antifolates.

While much effort has been devoted to understanding the biochemical pharmacology of antimalarial agents targeted at dihydrofolate reductase, the metabolically related thymidylate synthase activity has not been adequately exploited as a drug target. In *P. falciparum*, as in other protozoan parasites, thymidylate synthase activity is found on the same polypeptide that carries dihydrofolate reductase activity (2–4, 11, 17). The concept of inhibiting malarial thymidylate synthase is particularly attractive because, unlike mammalian cells, malarial parasites lack the ability to salvage preformed thymidine (27, 29). On this basis, a combination of a thymidylate synthase inhibitor and thymidine should have selective antimalarial activity. In principle, it would not be necessary to have a thymidylate synthase inhibitor that inhibits the parasite enzyme better than it does the mammalian enzyme, as long as the inhibitor is selective for thymidylate synthase and does not inhibit other functions in the cell.

Previous work from this laboratory centered around the antimalarial activity of 5-fluoroorotate (12, 23–26, 33). This compound inhibited the proliferation of malarial parasites at concentrations as low as 6 nM, whereas micromolar levels of the compound were required before toxicity was observed against mammalian cells. 5-Fluoroorotate appeared to exert its toxic effects on malarial parasites by undergoing metabolic conversion to 5-fluoro-2′-deoxyuridylate and subsequent inactivation of the parasite thymidylate synthase (26). It is important that thymidylate synthase inhibition in *P. falciparum* was found to be associated with cell death (33). As expected, pyrimidine nucleosides such as thymidine or uridine could not rescue malarial parasites from the toxic effects of 5-fluoroorotate. In mammalian cells, pyrimidine nucleosides could decrease the toxic effects of 5-fluoroorotate, but the decrease in toxicity was not complete, suggesting that the toxicity of 5-fluoroorotate in mammalian cells arises through a complex mechanism (12, 25).

In addition to 5-fluoropyrimidine-based inhibitors, thymidylate synthase can also be inhibited by folate-based inhibitors. Recent studies indicate that ICI D1694 (Fig. 1) is a potent inhibitor of mammalian cell proliferation (14). While the unmodified form of this compound is only a 25-fold better inhibitor of thymidylate synthase than of dihydrofolate reductase, its intracellular activity is thought to be targeted almost

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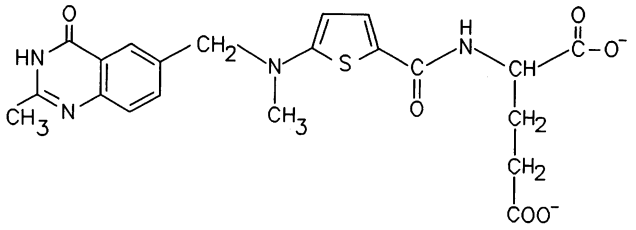

FIG. 1. Structure of ICI D1694.

exclusively at thymidylate synthase. This selective activity against thymidylate synthase is largely due to the intracellular conversion of ICI D1694 to a polyglutamylated derivative (14, 32). The latter is a 3,000 times better inhibitor of thymidylate synthase than of dihydrofolate reductase (14).

In order to determine whether folate-based inhibitors of thymidylate synthase can be used for selective malaria chemotherapy, the interactions of *P. falciparum*-infected erythrocytes with ICI D1694 are described in this report.

MATERIALS AND METHODS

Reagents. ICI D1694, also called *N*-{5-[*N*-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-*N*-methylamino]-2-thenoyl}-*S*-glutamic acid, was kindly provided by F. T. Boyle of ICI Pharmaceuticals, Cheshire, United Kingdom. RPMI 1640 cell culture medium, basal salt solution, amino acid solution, individual vitamins, and thymidine were purchased from Sigma Chemical Co. (St. Louis, Mo.). American Radiolabelled Chemicals (St. Louis, Mo.) supplied the [G-^{3}H]hypoxanthine (10 Ci/mmol). Human erythrocytes and plasma were obtained through the American Red Cross (Baltimore, Md.).

Parasites. The experiments described here were performed with the following isolates of *P. falciparum* (19, 21): clone D6 from Sierra Leone (susceptible to all drugs under consideration in the present study), clone W2 from Indochina (resistant to chloroquine, pyrimethamine, and cycloguanil), isolate FCR3 from The Gambia (pyrimethamine susceptible, but cycloguanil resistant), clone HB3 from Honduras (pyrimethamine resistant and only partially resistant to cycloguanil), and isolate V1/S from Vietnam (strongly resistant to pyrimethamine and cycloguanil). Parasites were cultured in vitro as described previously (13, 31).

Mammalian cells. The following mammalian cell lines were used: HeLa cells (human cervix epithelioid carcinoma), L1210 cells (mouse lymphocytic leukemia), HT 1080 cells (human fibrosarcoma), GM cells (normal human fibroblasts), and IMR 90 cells (human lung). Cells were cultivated in Eagle minimum essential medium supplemented with 5 to 10% fetal bovine serum.

Antiproliferation assays. ICI D1694 was tested for its antimalarial activity by using a traditional in vitro antiproliferation assay. Parasite-infected erythrocytes (0.5% parasitemia, 2% hematocrit) in 96-well plates were exposed to ICI D1694 for 48 h and then to radioactive hypoxanthine for 24 h (7). The amount of radioactivity in the precipitable material served as an index of cell proliferation. The proliferation of cells treated with ICI D1694 was compared with the proliferation of control cells that did not receive the thymidylate synthase inhibitor.

Mammalian cells were evaluated in a similar manner. The rapidly dividing HeLa cells and L1210 cells were set up at an initial density of 4,000 cells per 200-μl volume of each well of a 96-well plate. The other cells were set up at a density of 10,000 cells per well. After 24 h, 25 μl of ICI D1694 was introduced at various concentrations. Following 48 h of incubation with the antiproliferative agent, each well was pulsed with 0.5 μCi of radioactive hypoxanthine, and the amount of radioactivity in the precipitable material was determined.

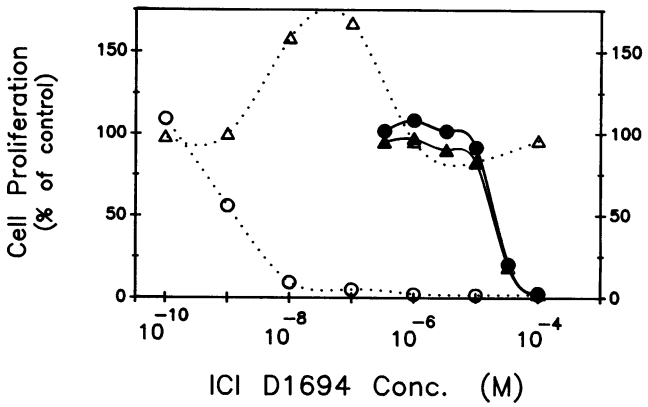

FIG. 2. Inhibition of *P. falciparum* proliferation and mouse L1210 proliferation after 48 h of exposure to ICI D1694 with and without thymidine. Closed circles, proliferation of the W2 clone of *P. falciparum* in the absence of thymidine; closed triangles, proliferation of *P. falciparum* parasites in the presence of 10 μM thymidine; open circles, proliferation of L1210 cells in the absence of thymidine; open triangles, proliferation of L1210 cells in the presence of 10 μM thymidine. Datum points are averages of four determinations at any given concentration of ICI D1694. Control parasites not treated with ICI D1694 incorporated 33,100 cpm of radioactive hypoxanthine metabolites into acid-precipitable material in the absence of thymidine and 37,800 cpm of hypoxanthine in the presence of 10 μM thymidine. Control L1210 cells incorporated 69,500 cpm of radioactivity in the absence of thymidine and 72,800 cpm in the presence of thymidine.

RESULTS

Antiproliferative activity of ICI D1694 against *P. falciparum*. Various concentrations of the quinazoline antifolate were incubated with parasite-infected erythrocytes for 48 h, after which parasitemia was estimated by hypoxanthine uptake (Fig. 2). Proliferation of W2 cells was inhibited by 50% with 20 μM ICI D1694. A full 100 μM was required to inhibit parasite proliferation by greater than 97%.

Antimalarial activity of ICI D1694 in the presence of thymidine. In a parallel study, *P. falciparum*-infected erythrocytes were treated with various doses of ICI D1694 in the presence of 10 μM thymidine (Fig. 2). Under these conditions, the amount of antifolate required to cause 50% inhibition of parasite proliferation (IC_{50}) was no different than that in the earlier case in which thymidine was not used in the culture medium.

Different isolates of *P. falciparum* show the same susceptibilities to ICI D1694. The in vitro susceptibilities of five different isolates of *P. falciparum* to ICI D1694 were examined (Table 1). The collection of isolates offered major variations in the active site of dihydrofolate reductase, clone HB3 was resistant to pyrimethamine but not cycloguanil, clone FCR3 was resistant to cycloguanil but not pyrimethamine, and clones W2 and V1/S were resistant to both types of dihydrofolate reductase inhibitors (21) (Table 1). All the isolates were equally susceptible to ICI D1694. These results are consistent with the hypothesis that the intracellular target of ICI D1694 is thymidylate synthase. In order to entertain an alternate hypothesis, that ICI D1694 binds to dihydrofolate reductase, one

TABLE 1. Comparison of the inhibitory activity of ICI D1694 against different isolates and clones of *P. falciparum* with and without thymidine

Isolate	Susceptibility (IC_{50} [μM])[a]						
	Pyrimethamine		Cycloguanil		ICI D1694		
					Without thymidine		With 10 μM thymidine and standard RPMI
	Standard RPMI	Special RPMI[b]	Standard RPMI	Special RPMI	Standard RPMI	Special RPMI	
D6	0.004	0.00012	0.00024	0.00012	25	25	40
FCR3	0.016	0.0022	0.255	0.215	45	110	55
HB3	1.05	0.089	0.008	0.0018	16	20	18
W2	16.5	0.137	0.255	0.0027	20	20	18
V1/S	8.06	0.339	3.98	0.096	30	25	30

[a] The IC_{50}s for pyrimethamine and cycloguanil susceptibilities were calculated from the published work of Peterson et al. (21). RPMI, RPMI 1640 culture medium.
[b] Special RPMI 1640 culture medium was prepared without folic acid and without *para*-aminobenzoic acid and was used with nondialyzed serum (18, 21).

would have to argue that binding interactions between ICI D1694 and the active site of dihydrofolate reductase involve amino acids other than those involved in pyrimethamine and cycloguanil resistance.

The susceptibility of *P. falciparum* to inhibitors of dihydrofolate reductase is known to depend on the folic acid and *para*-aminobenzoic acid concentrations in the RPMI 1640 culture medium (18, 21). Typically, the IC_{50}s of antifolates are 10-fold and sometimes as much as 100-fold lower when RPMI 1640 culture medium free of *para*-aminobenzoic acid and free of folic acid is used for the assay (18, 21) (Table 1). To determine whether the antimalarial activity of ICI D1694 was sensitive to different levels of vitamins in the medium, the antiproliferative activity of this compound was determined in modified RPMI 1640 culture medium. The antimalarial activity of ICI D1694 was independent of the vitamin status of the RPMI 1640 culture medium (Table 1).

Antiproliferative activity of ICI D1694 against mammalian cells. Jackman et al. (14) have shown that 100 nM ICI D1694 inhibits L1210 cells by 92% and that this inhibition can be reversed with 10 μM thymidine. However, in lieu of the poorer potency of ICI D1694 against malarial cells in culture, it was important to repeat the rescue experiment with higher amounts of ICI D1694. Figure 2 shows the dose-dependent inhibition of L1210 cells by ICI D1694 with and without thymidine. In the presence of 10 μM thymidine, low levels of ICI D1694 actually stimulated cell proliferation; even 100 μM ICI D1694 failed to inhibit the proliferation of L1210 cells.

It was important to determine whether the ability to utilize thymidine, for protection from ICI D1694 toxicity, was shared by mammalian cells other than L1210 cells. Parallel cytotoxicity studies were conducted on four additional mammalian cell lines (Table 2). The results showed that, in the absence of thymidine, mammalian cells varied slightly in terms of their vulnerability to ICI D1694. At the highest concentration tested (100 μM), some cells continued to incorporate nucleotides into their biopolymers. However, without exception, in the presence of thymidine, every cell line tested could overcome the cytotoxicity of 100 μM ICI D1694.

DISCUSSION

Design of new antimicrobial agents often relies on the identification and subsequent blockage of metabolic steps that are unique and essential to the survival of the infectious agent. In the case of malarial parasites, the enzyme thymidylate synthase is essential, since these parasites lack thymidine kinase activity and thus have no other means to obtain thymidylate for DNA synthesis (27, 29). In contrast, mammalian cells can use the pyrimidine nucleoside thymidine. In order to exploit this fundamental difference in pyrimidine metabolism between malarial and mammalian cells, it was important to identify an inhibitor of thymidylate synthase that was truly reaction specific; in other words, it was important to identify a compound that did not inhibit other reactions in the cell. ICI D1694 fit the requirements because not only was it shown in previous studies to be a potent inhibitor of thymidylate synthase but its antiproliferative activity against mammalian cells could also be reversed with exogenous thymidine (14).

As shown in the present study, a combination of thymidine and ICI D1694 was toxic to *P. falciparum* but not to mammalian cells. A concentration of 100 μM ICI D1694 completely inhibited the proliferation of parasites, but in the presence of 10 μM thymidine, ICI D1694 showed absolutely no toxicity to mammalian cells in culture. Selective inhibition of malarial parasite was possible, even though we started with a compound that was a 10,000-fold poorer inhibitor of *P. falciparum* proliferation than of mammalian cell proliferation. Our basic hypothesis for achieving selective chemotherapy was strongly supported by the present studies.

In principle, a concentration of 100 μM ICI D1694 in serum should allow for successful chemotherapy against malaria in animals. However, in practice, maintenance of such high concentrations of the compound in serum may be difficult. From a practical point of view, it was disappointing to learn that micromolar levels of ICI D1694 were required to success-

TABLE 2. Antiproliferative activity of ICI D1694 against mammalian cells can be completely reversed with thymidine

Cell line	Cell type	Susceptibility to ICI D1694 (IC_{50} [μM])[a]	
		Without thymidine	With thymidine[b]
HeLa	Human cervix epithelioid carcinoma	0.0002	>>100
L1210	Mouse lymphocytic leukemia	0.001	>>100
HT 1080	Human fibrosarcoma	5[a]	>>100
GM	Normal human fibroblasts	0.001[a]	>>100
IMR 90	Human lung	0.0001	>>100

[a] ICI D1694 was not particularly effective against HT 1080 and GM cell lines in the 48 h assay period; the proliferation rate was more than 50% even at the highest drug concentration tested. The values indicate the concentrations at which there was the most significant drop in proliferation rate.
[b] In the presence of thymidine, even at 100 μM ICI D1694, the proliferation rate of every cell line was comparable to that of control cells. Under these conditions, it was not possible to assign IC_{50}s of ICI D1694.

fully inhibit the proliferation of malarial parasites. At first, it was suspected that the lower degree of potency of ICI D1694 was due to the multiple-drug-resistant phenotype of the W2 clone or the abnormally high levels of folic acid and *para*-aminobenzoic acid in the culture medium. However, appropriate control experiments involving five different strains of *P. falciparum* and involving special RPMI 1640 culture medium allowed us to rule out the given explanations above for the poor antimalarial potency of ICI D1694. Other possible explanations include (i) poor binding of ICI D1694 to malarial thymidylate synthase, (ii) poor conversion of ICI D1694 to its pentaglutamate form in malarial parasites, (iii) poor enhancement of binding of ICI D1694 to malarial thymidylate synthase upon polyglutamylation, and (iv) poor transport of ICI D1694 into malarial cells. Experiments are under way to sort between these possibilities. The answer not only will lead us to selection of thymidylate synthase inhibitors that are better suited than ICI D1694 for malaria chemotherapy but may yield compounds that show preference for inhibiting parasites over mammalian cells in the absence of thymidine.

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