Inhibition of *Plasmodium falciparum* Dihydropteroate Synthetase and Growth In Vitro by Sulfa Drugs

YUN ZHANG¹ AND STEVEN R. MESHNICK^{2*}

Department of Microbiology, City University of New York Medical School, 138th Street at Convent Avenue, New York, New York 10031,² and Department of Biochemistry, City University of New York Graduate School and University Center, New York, New York 10036¹

Received 31 May 1990/Accepted 11 November 1990

The Michaelis-Menten inhibitory constants (K_s) and the concentrations required for 50% inhibition of the *Plasmodium falciparum* dihydropteroate synthetase were determined for six sulfa drugs. These drugs inhibited the in vitro growth of *P. falciparum* (50% lethal concentration) at concentrations of 30 to 500 nM; these concentrations were 100 to 1,000 times lower than the concentrations required for 50% inhibition and K_s (6 to 500 μ M). The uptake of *p*-aminobenzoic acid was not inhibited by the sulfa drugs. However, infected erythrocytes took up more labeled sulfamethoxazole than did uninfected erythrocytes. Thus, the concentration of sulfa drugs by malaria parasites may explain how sulfa drugs inhibit in vitro growth of parasites through the inhibition of dihydropteroate synthetase.

Antifolates are key antimalarial agents, particularly in the therapy and prophylaxis of chloroquine-resistant *Plasmodium falciparum*. Important antifolate antimalarial agents include pyrimethamine and chlorguanide, which inhibit dihydrofolate reductase, and sulfadoxine and dapson, which inhibit dihydropteroate synthetase (DHPS). Activities of combinations of inhibitors of both enzymes, such as sulfadoxine and pyrimethamine (Fansidar), are synergistic (17).

Currently, sulfadoxine-pyrimethamine is the drug of choice for the treatment of chloroquine-resistant malaria. Unfortunately, sulfadoxine-pyrimethamine cannot be used prophylactically because of its association with toxic epidermal necrolysis (Stevens-Johnson syndrome) (14). Furthermore, strains resistant to sulfadoxine-pyrimethamine have been isolated in Southeast Asia and Africa (5). Thus, there are serious limitations to the use of this agent in malaria control.

Sulfonamides and sulfones are selectively toxic to malarial parasites because their target enzyme, DHPS, is not present in the mammalian host. DHPS catalyzes the following step in the de novo biosynthesis of folic acid: *p*-aminobenzoic acid (PAB) + 6-hydroxymethyldihydropterin pyrophosphate $\rightarrow$ dihydropteroic acid. Sulfa drugs have been shown to inhibit DHPSs from the murine malarial parasites *Plasmodium berghei* (7, 9) and *Plasmodium chabaudi* (16). In vitro, sulfadoxine and dapson have been shown to inhibit *P. falciparum* growth and to act synergistically with pyrimethamine (4, 11). However, little is known about the relative potencies of various sulfa drugs against *P. falciparum*, either in terms of DHPS inhibition or their effectiveness in preventing growth of the parasite. The inhibition of *P. falciparum* growth in vitro by sulfa drugs is antagonized by PAB and folates (3, 18). The mechanism of antagonism, however, is not completely understood.

In this study we compared the effects of sulfa drugs on the in vitro growth of *P. falciparum* and *P. falciparum* DHPS enzyme activity. Evidence for the uptake of sulfa drugs by the parasites is presented. Inhibition of PAB uptake by sulfa

drugs as a possible alternative mechanism was also examined.

MATERIALS AND METHODS

Parasite cultures of *P. falciparum* FCR3 were maintained by the method of Trager and Jensen (15) in candle jars. The in vitro antimalarial activities of various sulfonamides were assessed by a modification of the method of Sixsmith et al. (13). Parasites were washed three times in warm PAB- and folate-free (PFF) RPMI 1640 medium (GIBCO, Grand Island, N.Y.) and were then cultivated for 72 h in the same medium containing 10% human serum (PFF-RS) prior to inoculation of microtiter plates. Parasites were inoculated (to a parasitemia level of 0.4% and a hematocrit of 1.5%) in 200 μ l of PFF-RS in 96-well microtiter plates (Corning, Corning, N.Y.). For determination of the concentrations which killed 50% of the parasites (LC_{50} s), 1:2 serial dilutions of drugs were made in triplicate wells. The final concentration of ethanol or dimethyl sulfoxide in each well was never greater than 1%, a concentration which does not affect parasite growth. After 48 h, [³H]hypoxanthine (0.5 μ Ci) was added to each well. After an additional 24 h, the microtiter plate was harvested by using a PHD cell harvester (Cambridge Technologies Inc., Cambridge, Mass.), and the filters were counted in Aquasol-2 (New England Nuclear, Boston, Mass.) in a liquid scintillation counter (LKB Rackbeta, Gaithersburg, Md.). LC_{50} s were determined from plots of incorporated counts versus drug concentrations. For each drug, the LC_{50} determination was performed a minimum of three times.

For measurement of DHPS activity, parasites were isolated from infected erythrocytes by saponin lysis (6) and stored at -70°C . The loss of the DHPS activity was negligible when isolated parasites were stored at -70°C for up to 6 months. Immediately prior to each experiment, thawed parasite preparations were lysed in Triton X-100 (0.05%) and microfuged for 10 min. Low-molecular-weight components were then removed by passing the supernatant through an NAP-5 gel filtration column (Pharmacia, Piscataway, N.J.).

The substrate of the synthetase reaction, 6-hydroxymethyldihydropterin pyrophosphate, was synthesized as de-

* Corresponding author.

scribed by Shiota et al. (12), except that the reaction of pyrophosphoric acid and pterin was incubated for 15 h instead of 3 h and the product was isolated by fast-performance liquid chromatography (Pharmacia) by using TSK-gel DEAE 650-S (Toyopearl, Gibbstown, N.J.) with a linear gradient of 50 to 110 mM LiCl.

The DHPS assay was performed by the method of Walter and Konigk (16) by measuring the incorporation of [14 C]PAB (56 μ Ci/ μ mol; Amersham, Arlington Heights, Ill.) into dihydropteroate. [14 C]dihydropteroate was separated from [14 C]PAB by using descending paper chromatography eluted with 0.1 M potassium phosphate (pH 7.0). Protein was assayed by the method of Bradford (2).

Michaelis-Menten constants (K_m s) were determined by using five substrate concentrations per experiment and averaging the K_m s that were calculated from at least two experiments. The concentrations required for 50% inhibition (IC_{50} s) were determined from plots of DHPS activities versus five drug concentrations under standard reaction condition. IC_{50} s of dapsone, sulfamoxole, sulfamethoxazole, and sulfaquinoxaline were determined by measuring the enzyme activities in the presence of each of these drugs at concentrations of 0, 50, 100, 200, and 500 μ M. The drug concentrations used to determine the IC_{50} s for sulfadoxine and sulfadiazine were 0, 200, 500, 1,000, and 2,000 μ M. Lineweaver-Burk plots were made for each drug, and inhibitory constants (K_i s) were determined by replotting apparent K_m s versus drug concentrations. At least four drug concentrations were used in each experiment. Plots were drawn by linear regression analysis by using Lotus 1-2-3, version 2, software. K_i determinations were repeated two to three times.

For uptake experiments, *P. falciparum*-infected erythrocytes and uninfected erythrocytes were incubated for 3 days in PFF-RS. To measure sulfamethoxazole uptake, infected erythrocytes were concentrated by gelatin flotation (6). Infected erythrocytes were then diluted with sham-incubated uninfected erythrocytes to the desired parasitemia levels. Cells were washed three times with warm PFF-RPMI 1640. Suspensions of 1 ml (5% hematocrit in PFF-RPMI 1640) were then incubated with 200 nM 3 H-labeled sulfamethoxazole (14.2 mCi/ μ mol; a gift from Peter Sorter, Hoffmann-La Roche, Nutley, N.J.). After 15 min, the cells were pelleted in a microfuge at 4°C for 1 min. The pellets were then washed three times with buffer (60 mM NaCl, 60 mM KCl, 1 mM NaH₂PO₄, 7 mM K₂HPO₄, 10 mM NaHCO₃, 11 mM glucose [pH 7.4]) containing 200 nM unlabeled sulfamethoxazole and lysed in 1 ml of water, which released the label from both erythrocytes and intracellular parasites. [14 C]PAB uptake experiments were performed similarly, except that the infected erythrocytes were not concentrated by gelatin flotation and the washing buffer contained 10 μ M PAB instead of sulfamethoxazole. The hemolysates were then transferred to 20-ml scintillation vials and treated with 1 drop of 1 M NaOH and 1 ml of 30% H₂O₂. After the color of hemoglobin was completely bleached, 18 ml of Aquasol-2 was added to each vial and radioactivity was counted in an LKB rackbeta liquid scintillation counter.

Aliquots of the erythrocyte suspensions used in uptake experiment were assayed for hemoglobin contents as described by Beutler (1). The number of erythrocytes in each uptake experiment was then calculated by assuming that 5×10^6 erythrocytes are equal to 360 μ g of hemoglobin (1).

TABLE 1. Inhibition of *P. falciparum* DHPS (IC_{50} and K_i) and parasite growth in vitro (LC_{50}) by six sulfa drugs

Drug	LC_{50} (nM) ^a	IC_{50} (μ M) ^b	K_i (μ M) ^c
Dapsone	31 $\pm$ 14	33	6 $\pm$ 2
Sulfamoxole	61 $\pm$ 17	55	7 $\pm$ 3
Sulfamethoxazole	60 $\pm$ 21	100	36 $\pm$ 19
Sulfaquinoxaline	519 $\pm$ 443	40	15 $\pm$ 3
Sulfadiazine	125 $\pm$ 25	500	89 $\pm$ 18
Sulfadoxine	357 $\pm$ 33	400	39 $\pm$ 24

^a Values are means $\pm$ standard deviations.

^b Values are averages of two to three determinations.

^c Determined by replotting apparent K_m s versus drug concentrations, given as y intercepts $\pm$ standard errors.

RESULTS

All six drugs tested exhibited antimalarial activity in vitro. The LC_{50} s of the drugs ranged from 31 to 520 nM (Table 1). Dapsone, sulfamoxole, and sulfamethoxazole were most effective, sulfadiazine and sulfadoxine had intermediate activities, and sulfaquinoxaline was least effective.

The inhibitory effects of these drugs on the *P. falciparum* DHPS were then studied. *P. falciparum* extracts contained an average of 10 μ U of DHPS activity per mg of protein. Apparent K_m s for this activity were 17.2 ± 1.3 μ M for 6-hydroxymethyldihydropterin pyrophosphate and 8.1 ± 2.9 μ M for PAB.

All five sulfonamides and dapsone were found to be competitive inhibitors of the *P. falciparum* DHPS activity (Fig. 1). The K_i of each sulfa drug is listed in Table 1. There was no apparent correlation between the ability of a drug to inhibit the parasite DHPS and its in vitro antimalarial activity (R^2 , 0.009 and 0.002 for IC_{50} s and K_i s, respectively).

Surprisingly, the drugs appeared to inhibit parasite growth in vitro at concentrations 100- to 1,000-fold lower than their K_i s and IC_{50} s. This suggested that the sulfa drugs either act by an additional mechanism or that they are concentrated by the parasites.

Since malaria parasites require PAB, we suspected that sulfa drugs might inhibit parasite growth by inhibiting PAB uptake. The uptake of 1 to 10 μ M [14 C]PAB was the same in the presence and absence of 100 μ M sulfamethoxazole (Fig. 2). Similar results were obtained by using 100 μ M sulfadiazine and sulfaquinoxaline (data not shown). In contrast, 100 μ M cold PAB completely blocked uptake (Fig. 2).

To determine whether parasites concentrate sulfa drugs, sulfamethoxazole uptake by infected and normal erythrocytes was studied. Sulfamethoxazole was taken up by both infected and normal erythrocytes within 1 min (data not shown). Infected erythrocytes were found to take up significantly more sulfamethoxazole than that taken up by normal erythrocytes (Fig. 3). The degree of uptake was proportional to the parasitemia ($R^2 = 0.88$). By subtracting the [3 C]sulfamethoxazole content in uninfected erythrocytes from that predicted in 100% infected erythrocytes, the intraparasitic [3 C]sulfamethoxazole content was estimated to be 0.06 nmol/ 10^{10} cells.

DISCUSSION

In this study, the efficacies of six sulfa drugs as inhibitors of the *P. falciparum* DHPS and of the organism's growth in vitro were measured. These drugs exerted their toxic effects on the parasites at concentrations 100- to 1,000-fold lower than the concentrations which inhibited the enzyme. This

FIG. 1. Lineweaver-Burk plots of inhibition of *P. falciparum* DHPS by six sulfa drugs. DHPS activities were measured under standard assay conditions in the presence of various drug concentrations.

difference was not due to inhibition of PAB uptake. However, infected erythrocytes were found to concentrate a radiolabeled sulfonamide. Therefore, the intraparasitic concentrations of the drugs could be much higher than the extracellular concentrations and could perhaps be high enough to inhibit the parasite's DHPS.

The susceptibility of cultured *P. falciparum* to sulfadoxine, the sulfa component of sulfadoxine-pyrimethamine, has been examined previously. The LC_{50} of sulfadoxine reported here (360 nM) is in the same range as that reported for other sulfadoxine-pyrimethamine-susceptible strains (900 and 290 nM, in references 3 and 17, respectively). In this study, we examined the susceptibilities of *P. falciparum* in vitro to an additional five sulfa drugs, some of which are much more effective in inhibiting parasite growth (Table 1).

DHPS has been studied in *Escherichia coli* (8), *Lactobacillus plantarum* (12), *P. chabaudi* (16), *P. berghei* (7, 9), and most recently, *Pneumocystis carinii* (10). The specific activities of *P. falciparum* crude extracts reported here (10

$\mu\text{U}/\text{mg}$ of protein) were much lower than those reported for *E. coli* (400 $\mu\text{U}/\text{mg}$ of protein [10]) and *L. plantarum* (720 $\mu\text{U}/\text{mg}$ of protein [12]) but were in the same range as those reported for *P. chabaudi* (16 $\mu\text{U}/\text{mg}$ of protein [16]) and *P. carinii* (14 $\mu\text{U}/\text{mg}$ of protein [10]). The K_m s for PAB and hydroxymethyldihydropterin pyrophosphate in *P. falciparum* reported here (8.1 and 17.2 μM , respectively) are higher than those reported in *P. berghei* (2.8 and 1.4 μM , respectively [9]), which were measured by using partially purified enzyme.

As expected, all sulfa drugs studied inhibited DHPS by competing with PAB (Fig. 1). Surprisingly, however, a 2 to 3 order of magnitude difference was seen between the concentrations which inhibited parasite growth and concentrations which inhibited the parasite DHPS. A similar difference has not been reported for malaria or any other protozoa parasites. This suggests either that sulfa drugs act by a mechanism other than DHPS inhibition or that the sulfa drugs are concentrated by malaria parasites.

FIG. 2. Competition of [¹⁴C]PAB uptake by unlabeled PAB and sulfamethoxazole. □, [¹⁴C]PAB alone; ◆, [¹⁴C]PAB plus 100 μM sulfamethoxazole; ■, [¹⁴C]PAB plus 100 μM unlabeled PAB.

We showed in this study that infected erythrocytes take up significantly more sulfamethoxazole than do uninfected erythrocytes. Sulfamethoxazole uptake was proportional to parasitemia, indicating that this uptake is induced by the parasites (Fig. 3). The conditions used for the uptake study were physiologically relevant, since the drug concentration used (200 nM) was close to its effective antimalarial concentration.

Although no correlation was seen between the LC₅₀s and the IC₅₀s or K_s for the six drugs tested, if sulfaquinoxaline and sulfadiazine are excluded, then there was a good correlation for the remaining four drugs ($R^2 = 0.980$ for IC₅₀s). The fact that sulfadiazine is a better antiplasmodial agent than a DHPS inhibitor is not, however, due to its ability to inhibit PAB uptake (see Results).

Could the measured drug uptake explain why the LC₅₀s of the sulfa drugs were so much lower than their K_s? The intraparasitic concentrations of the sulfa drugs were not

FIG. 3. Parasitemia dependence of [³H]sulfamethoxazole uptake by infected erythrocytes. Radioactivities associated with erythrocytes were measured after incubating 5% of the erythrocytes with different parasitemia levels with 200 nM [³H]sulfamethoxazole for 15 min at 37°C.

measured directly because of the inability to maintain malaria parasites extracellularly. By assuming that the concentration of sulfamethoxazole in the erythrocytic cytoplasm of infected erythrocytes is the same as that in the cytoplasm of uninfected erythrocytes and that mature parasites are spherical with a radius of 2.5 μm (5), we calculated the intraparasitic concentration of sulfamethoxazole. The radioactivity associated with uninfected erythrocytes was subtracted from the radioactivity associated with the same number of infected erythrocytes (corrected to 100% parasitemia); the difference was then divided by the total volume of the parasites, giving an intraparasitic concentration of 4.3 μM. This was 20 times higher than the drug's extracellular concentration, which was still considerably lower than the 100- to 1,000-fold difference observed between the concentration that inhibited parasite growth and the concentration that inhibited DHPS. There are three explanations for this. First, our estimates of parasite size or the concentration of drug in the infected erythrocyte cytoplasm may have been too high. Second, the microenvironment of the enzyme inside parasites is different than that in the enzyme assay solution, and the enzyme may have different susceptibilities to the sulfa drugs. Third, partial inhibition of DHPS activity may be lethal to the organism. Attempts are under way to better characterize this uptake and concentration of sulfa drugs by parasites. These findings could aid in the development of better antimalarial therapies.

ACKNOWLEDGMENTS

This research was supported by a grant from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases and by Public Health Service grants AI-26848 and RR-03060 from the National Institutes of Health.

We thank Allen Ranz for technical assistance and Peter Sorter of Hoffmann-La Roche for providing compounds.

REFERENCES

- Beutler, E. 1984. Red cell metabolism, a manual of biochemical methods, 3rd ed., p. 12. Grune & Stratton, Inc., Orlando, Fla.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.
- Brockelman, C. R., and P. Tan-Ariya. 1982. *Plasmodium falciparum* in continuous culture: a new medium for the *in vitro* test for sulfadoxine sensitivity. *Bull. W.H.O.* 60:423-426.
- Chulay, J. D., W. M. Watkins, and D. J. Sixsmith. 1984. Synergistic antimalarial activity of pyrimethamine and sulfadoxine against *Plasmodium falciparum* *in vitro*. *Am. J. Trop. Med. Hyg.* 33:325-330.
- Dieckmann, A., and A. Jung. 1986. Mechanisms of sulfadoxine resistance in *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* 19:143-147.
- Fairfield, A. S., S. R. Meshnick, and J. W. Eaton. 1983. Malaria parasites adopt host superoxide dismutase. *Science* 221:764-769.
- Ferone, R. 1973. The enzymatic synthesis of dihydropteroate and dihydrofolate by *Plasmodium berghei*. *J. Protozool.* 20: 459-464.
- McCullough, J. L., and T. H. Maren. 1973. Inhibition of dihydropteroate synthetase from *Escherichia coli* by sulfones and sulfonamides. *Antimicrob. Agents Chemother.* 3:665-669.
- McCullough, J. L., and T. H. Maren. 1974. Dihydropteroate synthetase from *Plasmodium berghii*: isolation, properties, and inhibition by dapsone and sulfadiazine. *Mol. Pharmacol.* 10: 140-145.
- Merali, S., Y. Zhang, D. Sloan, and S. Meshnick. 1990. Inhibition of *Pneumocystis carinii* dihydropteroate synthetase by sulfa drugs. *Antimicrob. Agents Chemother.* 34:1075-1078.
- Scott, H. V., K. H. Rieckmann, and W. J. O'Sullivan. 1987.

- Synergistic antimalarial activity of dapsone/dihydrofolate reductase inhibitors and interaction of antifolate, antipyrimidine and antipurine combinations against *Plasmodium falciparum* in vitro. Trans. R. Soc. Trop. Med. Hyg. **81**:715-721.
12. Shiotani, T., M. N. Disraeli, and M. P. McCann. 1963. The enzymatic synthesis of folate-like compounds from hydroxymethyl-dihydropteridine pyrophosphate. J. Biol. Chem. **239**: 2259-2266.
 13. Sixsmith, D. G., W. M. Watkins, J. D. Chulay, and H. C. Spencer. 1984. In vitro antimalarial activity of tetrahydrofolate dehydrogenase inhibitors. Am. J. Trop. Med. Hyg. **33**:772-776.
 14. Stitt, V. J., Jr. 1988. Stevens-Johnson syndrome: a review of the literature. J. Natl. Med. Assoc. **80**:104-108.
 15. Trager, W., and J. B. Jensen. 1976. Human malaria parasites in continuous culture. Science **193**:673-675.
 16. Walter, R., and D. E. Konigk. 1980. 7,8-Dihydropteroate-synthesizing enzyme from *Plasmodium chabaudi*. Methods Enzymol. **66**:564-569.
 17. Warhurst, D. C. 1987. Antimalarial drugs: an update. Drugs **33**:50-65.
 18. Watkins, W. M., D. G. Sixsmith, J. D. Chulay, and H. C. Spencer. 1985. Antagonism of sulfadoxine and pyrimethamine antimalarial activity in vitro by p-aminobenzoic acid, p-aminobenzoylglutamic acid and folic acid. Mol. Biochem. Parasitol. **14**:55-61.