2,4-Diaminopteridine-Based Compounds as Precursors for De Novo Synthesis of Antifolates: a Novel Class of Antimalarials

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We have tested the hypothesis that 2,4-diamino-6-hydroxymethyl-pteridine (DAP), 2,4-diaminoptericoic acid (DAPA), and 2,4 diamino-N10-methyl-ptericoic acid (DAMPA) could be converted into aminopterine (from DAP and DAPA) and methotrexate (from DAMPA), both of which are potent inhibitors of dihydrofolate reductase, a proven drug target for *Plasmodium falciparum*. DAP, DAPA, and DAMPA inhibited parasite growth in the micromolar range; DAMPA was the most active, with 50% inhibitory concentrations in vitro of 446 ng/ml against the antifolate-sensitive strain and 812 ng/ml against the highly resistant strain under physiological folate conditions. DAMPA potentiates the activity of the sulfone dapsone, an inhibitor of dihydropteroate synthase, but not that of chlorcycloguanil, a known inhibitor of dihydrofolate reductase (DHFR). Experiments with a *Saccharomyces cerevisiae* strain dependent upon the *P. falciparum* DHFR enzyme showed that DHFR is a target of DAMPA in that system. We hypothesize that DAMPA is converted to methotrexate by the parasite dihydrofolate synthase, which explains the synergy of DAMPA with dapsone but not with chlorcycloguanil. This de novo synthesis will not occur in the host, since it lacks the complete folate pathway. If this hypothesis holds true, the de novo synthesis of the toxic compounds could be used as a framework for the search for novel potent antimalarial antifolates.

Chemotherapy remains one of the most important tools for the management of falciparum malaria. However, malaria control is hampered by the emergence and spread of parasites resistant to almost all available antimalarial drugs. This situation is critical in Africa as a result of the spread of resistance to the combination sulfadoxine-pyrimethamine, an inexpensive treatment widely used in African countries (9, 16–18, 25, 28). As an alternative, a number of combinations with artemisinins are being recommended and implemented, but questions about the cost and the adequacy of the supply of artemisinins and the intrinsic ability of *Plasmodium falciparum* to select drug-resistant parasite populations underline the need to identify novel agents.

Bacteria, plants, and many unicellular eukaryotic organisms depend upon the de novo synthesis of dihydrofolate (DHF), a key cofactor in the biosynthesis of thymidine. In contrast, multicellular animals depend on the uptake of preformed folate in food. This difference has been exploited for more than 50 years to design drugs that inhibit folate synthesis in bacteria and protozoan pathogens like *P. falciparum* but that have little effect on the human host (10). For example, sulfa drugs inhibit dihydropteroate synthase (DHPS; EC 2.5.1.15), an enzyme needed for folate synthesis, and specifically deprive the pathogen of DHF and thus inhibit DNA synthesis (20). Despite the importance of this critical pathway, efforts to target folate

metabolism have been restricted to sulfa drugs that inhibit DHPS or competitive inhibitors of dihydrofolate reductase (DHFR; EC 1.5.1.3), an enzyme required for cyclic utilization of the folate cofactor. Study of the folate pathway shows that other enzymes within this pathway might be exploited as therapeutic targets (14, 23), but there have been only limited attempts to do so.

Aminopterine and methotrexate are potent inhibitors of virtually all DHFR enzymes, including the DHFR enzyme of humans, and both drugs are used for the treatment of diverse malignancies (3). In vitro studies have shown that aminopterine and methotrexate are also potent inhibitors of *P. falciparum* growth (6, 8, 29). However, methotrexate inhibits both parasite growth and the division of neoplastic cells in the same concentration range (21). Therefore, these compounds cannot be used directly to treat malaria because of their narrow therapeutic indices and the resulting life-threatening toxicity to the human host.

Based on this information, we have hypothesized that precursors of methotrexate or aminopterine might be used in humans to safely synthesize these potent inhibitors within the parasite cells. By this logic, when the parasite is supplied with 2,4-diamino-6-hydroxymethyl-pteridine (DAP), 2,4-diaminoptericoic acid (DAPA), or 2,4 diamino-N10-methyl-ptericoic acid (DAMPA) (Fig. 1 and 2), the parasite would synthesize aminopterine (from DAP and DAPA) and methotrexate (from DAMPA) de novo. One of these precursors, DAMPA, has been shown to be inactive against mammalian cells and well tolerated in nonhuman primates (33), so this approach would allow the toxic compounds to be synthesized only within *P. falciparum* and to target specifically the parasite DHFR.

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FIG. 1. Chemical structures of DHF, methotrexate, aminopterin, DAP, DAPA, and DAMPA.

Methotrexate is a particularly potent drug for at least two main reasons. First, it is only a slight modification of the normal substrate of DHFR, DHF (Fig. 1), so it competes effectively with the substrate in the DHFR active site. Second, like DHF, methotrexate and aminopterin support the addition of extra glutamate residues by folylpolyglutamate synthase (FPGS; EC 6.3.2.17), and the polyglutamation extends the range of targets for these compounds (1, 5). These observations indicate that, like methotrexate, DAP, DAMPA, and DAPA may target other enzymes in this key set of biosynthetic pathways. In this paper, we report on the initial steps required

to assess the effects of the diaminopteridine compounds DAP, DAPA, and DAMPA alone or in combination with the DHFR inhibitor chlorcycloguanil and the DHPS inhibitor dapsone on the in vitro growth of *P. falciparum* and a *Saccharomyces cerevisiae* strain expressing the *Plasmodium* DHFR enzyme.

MATERIALS AND METHODS

The compounds DAP, DAPA, DAMPA, pyrimethamine, dapsone, sulfanilamide, dTMP, dextrose, dimethyl sulfoxide (DMSO), and [^3H]hypoxanthine were purchased from Sigma, United Kingdom. Chlorcycloguanil was a gift from

FIG. 2. Folate pathway in *Plasmodium falciparum* and the proposed mode of action of the diaminopteridines, analogs of folate precursors. Known inhibitions are shown with solid lines, while postulated additional inhibitions are shown with dotted lines. Abbreviations: HMP, hydroxy-methyl-pteridine; HMP-PP, hydroxy-methyl-pteridine pyrophosphate; DHP, dihydropteroate; DHF-Glu(n), dihydrofolate polyglutamate; THF-Glu(n), tetrahydrofolate polyglutamate; meTHF-Glu(n), methylene-tetrahydrofolate polyglutamate; MTX, methotrexate; AMP, aminopterin; DHNA, dihydroneopterin aldolase; PPPK, dihydroneopterin pyrophosphokinase; DHFS, dihydrofolate synthase; TS, thymidylate synthase; SHMT, serine-hydroxy-methyltransferase.

AstraZeneca, United Kingdom. Yeast extract was purchased from Becton Dickinson Microbiology Systems.

Drug assay in *P. falciparum*. Antimalarial activity was measured in the presence of various concentrations of each compound by radioisotope incorporation (27). The results were expressed as the drug concentration required for 50% inhibition of [^3H]hypoxanthine incorporation into parasite nucleic acid (IC_{50}) by nonlinear regression analysis of the dose-response curve. Two reference *P. falciparum* laboratory isolates were tested: M24, a fully pyrimethamine-sensitive isolate, and V1/S, a highly pyrimethamine-resistant isolate (31). M24 carries a wild-type *dhfr* gene; but the V1/S isolate has four mutations in its *dhfr* gene, at codons 108, 51, 59, and 164. Two kinds of culture medium, RPMI 1640 (GIBCO BRL, United Kingdom), were employed. One contained *para*-aminobenzoic acid (pABA) and folic acid at physiological concentrations (0.5 $\mu\text{g}/\text{liter}$ and 10 $\mu\text{g}/\text{liter}$, respectively), and the other contained neither folate nor pABA. RPMI 1640 was supplemented with 10% (vol/vol) normal human serum, 25 mM bicarbonate, and 25 mM HEPES buffer.

Synergy was measured in vitro both geometrically, by construction of isobolograms (with a minimum of five coordinates), and algebraically, by calculation of the sum of the minimum fractional inhibitory concentrations (FICs) (2). Synergy is demonstrated when the FIC is <0.5 . An FIC value of >4.0 denotes an antagonistic effect, and an FIC value between 0.5 and 4 indicates either a nonsynergistic or a nonantagonistic interaction.

Drug assays with *Saccharomyces cerevisiae*. The TH5 strain of *Saccharomyces cerevisiae* (*MATa leu2-3,112 trp1 ura3-52 dfr1::URA3 tup1*) lacks endogenous DHFR activity (11). As described previously (35), the *dhfr*-deficient yeast strain was used to express *Plasmodium falciparum* DHFR (pDHFR) of fully antifolate-sensitive laboratory reference isolate D6 (wild-type DHFR) and the highly pyrimethamine-resistant isolate V1/S (51I/59R/108N/164L). *Dfr1* mutant TH5 yeast cells transformed with the yeast DHFR (yDHFR) were also constructed and used as a control. For all experiments, yeast strains were grown at 30°C in either rich (YEPD) or selective (without tryptophan) medium. The growth of TH5 before transformation requires supplementation of the medium with 100 $\mu\text{g}/\text{ml}$ of dTMP (Sigma, St. Louis, MO).

The drug sensitivity assays were performed as described previously to obtain quantitative measures of drug sensitivity (24). Briefly, the growth of the yeast in this assay depends upon the antifolate resistance of the *dhfr* allele expressed (35). The yeast was grown in 96-well plates at the indicated concentrations of the drugs dissolved in DMSO. To increase the sensitivity of the yeast to DHFR inhibitors, both the control and the drug-containing wells contained 1 mM sulfanilamide (13). The growth of the yeast in each well was assessed by reading the optical density at 650 nm after approximately 24 h of incubation at 30°C, and the growth in any well was compared with the growth of that strain in solvent alone. The numerical IC_{50} value was calculated from the slope and the intercept of the line

TABLE 1. In vitro activities of DAP, DAPA, and DAMPA against reference *Plasmodium falciparum* isolates M24 and V1/S^a

Drug	IC ₅₀ (ng/ml) for M24 in:		Increase factor for M24 ^b	IC ₅₀ (ng/ml) for V1/S in:		Increase factor for V1/S
	Minimum folate	Physiological folate		Minimum folate	Physiological folate	
DAP	3,319 ± 179	17,100 ± 11,253	5.2	7,885 ± 653.48	39,950 ± 22,083	5.1
DAPA	248.24 ± 77	1,132 ± 1,061	4.6	38,694 ± 9,122	47,035 ± 10,196	1.21
DAMPA	17.97 ± 3.6	248.05 ± 75	13.8	445.78 ± 144	812.47 ± 324	1.8
CCG	0.06 ± 0.03	0.09 ± 0.03	1.5	2.9 ± 2.5	20 ± 2	7
PM	0.25 ± 0.025	0.99 ± 0.74	4.0	213 ± 149	1,042 ± 223	4.9
DDS	22.34 ± 17	8,325 ± 3,044	372.6	3,975 ± 3,427	19,318 ± 7,946	4.9

^a Dapsone (DDS), chlorcycloguanil (CCG), and pyrimethamine (PM) were used as reference antifolates. The culture medium composition is as described in Materials and Methods.

^b Increase factor is defined as the ratio of the IC₅₀ in physiological folate to the IC₅₀ in minimum folate (folate-free medium).

defined by the two datum points that bracket 50% relative growth. IC₅₀ assays were performed at least twice for each allele to ensure reproducibility.

RESULTS AND DISCUSSION

Our first goal was to compare the effectiveness of DAP, DAPA, and DAMPA with that of dapsone, pyrimethamine, and chlorcycloguanil on the growth of pyrimethamine-sensitive and -resistant *P. falciparum* isolates. The structures of these compounds are shown in Fig. 1. Folate metabolism and the postulated pathways and targets of DAP, DAPA, and DAMPA are shown schematically in Fig. 2. Parasite growth inhibition was assessed in two ways. First, pyrimethamine-sensitive isolate M24 and pyrimethamine-resistant isolate V1/S were grown and tested in physiological folate. These data are summarized in Table 1. The IC₅₀ values for both the pteridines (DAP, DAPA, and DAMPA) and the reference compounds (chlorcycloguanil, pyrimethamine, and dapsone) were lower against M24, the fully sensitive isolate, than against antifolate-resistant isolate V1/S. These data indicate that these compounds bear some antimalarial activity, with DAMPA being the most active; DAMPA showed IC₅₀ values of about 446 ng/ml against the sensitive isolate and 812 ng/ml against the V1/S isolate.

Second, we compared the growth inhibition of both parasites in minimum folate medium. Under these conditions, the activities of antifolates should increase because the parasite cannot salvage exogenous folate to bypass the antifolate effect (30–32). As predicted, the IC₅₀ values for all compounds were decreased in comparison with those obtained in physiological folate, and most were decreased by at least a factor of 4 (Table 1).

These data support the hypothesis that DAP, DAPA, and DAMPA target the folate pathway. The V1/S parasite carries a highly mutant allele of the *dhfr* gene (51I/59R/108N/164L), and the marked decreases in the potencies of pyrimethamine and chlorcycloguanil result from diminished binding of the drugs to that enzyme (26). Thus, the increased IC₅₀ values for the pteridine compounds against V1/S suggest that they may also target DHFR. It is interesting that the difference between the wild-type and the mutant strain values for pyrimethamine was about 1,000-fold, as has been documented previously (Table 1), but the corresponding difference for DAMPA was less than 25-fold. This fairly modest increase in resistance to DAMPA by the highly mutant DHFR enzyme highlights a potential advantage of this class.

To study further the modes of action of these compounds,

we assessed their effects on the in vitro growth of *S. cerevisiae* cells that lack endogenous DHFR activity and that depend upon a *dhfr* allele derived from *P. falciparum* for growth. First, yeast cells that expressed the pyrimethamine-sensitive allele were grown on plates to assess their sensitivities to DAP, DAPA, and DAMPA. No inhibitory activity was observed when DAP and DAPA were used at concentrations up to 1,910 µg/ml and 3,330 µg/ml, respectively, the maximum concentrations that can be reached in DMSO (data not shown). However, DAMPA had inhibitory activity when the concentration of DAMPA in the plates was 360 µg/ml. Thus, further experiments were carried out with DAMPA.

We then assessed the inhibition profile of yeast lines dependent upon the wild-type or the highly mutant allele of *pfdhfr*. Cells were grown in liquid medium with 1 mM sulfanilamide and DAMPA at concentrations ranging from 0 to 72,000 ng/ml. Figure 3 summarizes these data. The IC₅₀ value of DAMPA against the wild-type enzyme was 400 ng/ml, and that against the quadruple mutant was 16,000 ng/ml. These substantial increases in the IC₅₀s for yeasts expressing wild-type and mutant *pfdhfr* parallel the results for the M24 and V1/S isolates. As a control, the isogenic yeast line dependent upon the yeast DHFR enzyme was also tested, and no growth inhibition was observed. The differential inhibition of the wild-type and mutant *P. falciparum* enzymes and the complete lack of inhibition of the yeast enzyme demonstrate that DAMPA or its metabolite could target *Plasmodium* DHFR.

TABLE 2. In vitro activities of the combinations of DAMPA with dapsone and of DAMPA with chlorcycloguanil against the *Plasmodium falciparum* V1/S isolate

Drug	Drug IC ₅₀ (µM) in presence of ^a :		Summed FIC
	DAMPA	DDS (ng/ml)	
CCG	15.2 (90) ^b		0.86
	17.2 (237)		1.00
	1.4 (719)		0.95
	2.9 (611)		0.89
DAMPA		129 (2,518)	0.29
		143 (3,490)	0.36
		104 (5,388)	0.41

^a The IC₅₀s of DAMPA, chlorcycloguanil (CCG), and dapsone (DDS) alone were 812, 20, and 19,566 ng/ml respectively.

^b Values in parentheses represent the concentration of DAMPA or dapsone (in ng/ml).

FIG. 3. Effect of DAMPA on growth of *Saccharomyces cerevisiae* expressing the *Plasmodium falciparum* wild type or mutant dihydrofolate reductase gene (*dhfr*). *S. cerevisiae* expressing its own *dhfr* gene (isogenic cell) was used as a control. IC_{50} s are in ng/ml.

It has been known for more than 50 years that the combination of a sulfa drug and an inhibitor of DHFR produces a synergistic inhibition of the folate pathway (4). Pyrimethamine and sulfadoxine are combined in the antimalarial Fansidar to take advantage of this effect, and chlorproguanil (the prodrug of chlorcycloguanil) has been formulated with dapsone to produce the new drug Lapdap (34). We hypothesize that DAMPA and DAPA are metabolized to methotrexate and aminopterin, respectively, by the mediation of dihydrofolate synthase (EC 6.3.2.12) and that these compounds would further be polyglutamated in the presence of FPGS. These mono- and polyglutamated metabolites would be expected to inhibit the parasite DHFR. Under these conditions, DAMPA and DAPA would not act synergistically with DHFR inhibitors because they affect a common target, DHFR, but would act synergistically with sulfa drugs.

To test this hypothesis, the V1/S isolate was grown in the presence of changing ratios of DAMPA and dapsone or chlorcycloguanil. The results are summarized in Table 2. The minimum FICs of the combination of DAMPA and dapsone were less than 0.5, a clear indication that DAMPA interacts synergistically with dapsone. However, FICs representing the IC_{50} of DAMPA in the presence of chlorcycloguanil are between 0.85 and 0.9, indicating that these two compounds act either nonsynergistically or nonantagonistically. Both results support the idea that DAMPA or its metabolites functions as an inhibitor of DHFR.

Two possible mechanisms could explain the activity of DAMPA. DAMPA could act directly on DHFR, or it may be metabolized to methotrexate, which then targets DHFR. In fact, both mechanisms may be operating, and biochemical

analysis will be required to identify the actual mechanism. However, we favor the hypothesis that DAMPA is metabolized to methotrexate, similar to the de novo synthesis of the pteridine-sulfa drug from the sulfa drug in *P. falciparum* (19).

In mammalian cells, methotrexate is polyglutamated in vivo by the enzyme FPGS, and this increases the activity of methotrexate against DHFR (7, 15, 22). Moreover, polyglutamation converts methotrexate into a potent inhibitor of other enzymes in the folate pathways, such as thymidylate synthase, and enzymes that use folate derivatives in the purine synthesis pathway (1, 5). The polyglutamated forms of folate derivatives have been identified in *P. falciparum* (12), and the polyglutamation is mediated by a single enzyme that has activities against both dihydrofolate synthase and folypolyglutamate synthase (14, 23). We therefore predict that methotrexate and aminopterin synthesized in situ will be converted to their polyglutamated forms, greatly broadening their modes of action (1, 5). This broader activity would differ from that of current DHFR inhibitors used for the treatment of apicomplexan and bacterial infections. Furthermore, the evolution of resistance to a compound that can affect more than one target would presumably be more difficult.

Thus, if the de novo synthesis of toxic compounds is proven true, this information could be used as a framework for the search for novel antifolates with activities against a wider range of prokaryotic and eukaryotic pathogens. Such antifolates would be the more interesting because the de novo synthesis of toxic compounds will not occur in the host because it lacks the complete folate pathway. Studies aimed at identifying and characterizing DAMPA toxic metabolites are under way.

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