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THE MALARIAL FOLATE PATHWAY AND MOLECULAR TARGETS FOR ANTIMALARIAL DEVELOPMENT

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ABSTRACT

The malarial folate pathway is distinct from that of the host, and contains enzymes which are proven or potential targets for antimalarial drugs. Plasmodium falciparum *contains both the* de novo *synthesis and salvage pathways leading to tetrahydrofolylpolyglutamate, which joins two cycles sharing a common step. One cycle is the thymidylate synthesis cycle, and the other the methionine synthesis cycle. While the function of the former is to supply thymidylate required for DNA synthesis, that of the latter appears to be, not to supply methionine but to generate active folate cofactors from more stable precursors salvaged by the parasites. We have designed and made a synthetic gene coding for* P. falciparum *bifunctional enzyme in the thymidylate synthesis cycle, namely, dihydrofolate reductase-thymidylate synthase (DHFR-TS), which is the target for such drugs as pyrimethamine and cycloguanil. The aim is to obtain large amounts of the enzyme from the expression of the synthetic gene, which otherwise cannot be obtained from the parasite. We have been able to express large amounts of the malarial DHFR from* E. coli, *and have found that the activities of several antifolates against the enzyme parallel their antimalarial activities. This enabled us to screen candidate antimalarials directed against this target. The synthetic gene also allows us to make a number of mutant enzymes. We show that mutation at specific sites, earlier known to produce drug-resistant parasites, leads to weaker binding with pyrimethamine and cycloguanil as expected. From the study of various mutant enzymes, both found and not found in nature, we show that there is correlation between the binding affinities of pyrimethamine and cycloguanil, and raise doubt to the current hypothesis that there is little cross resistance between pyrimethamine and cycloguanil.*

INTRODUCTION

Malaria continues to be a major public health problem, especially for a tropical developing country like Thailand. The problem is compounded by the fact that the agent for falciparum malaria, *Plasmodium falciparum*, has become widely resistant to most antimalarial drugs. While there is as yet no vaccine for this disease, it is necessary to look for new antimalarials which should be both effective and cheap. Since the majority of the people at risk are poor, the market for new antimalarials is not commercially attractive and most drug companies do not have active programmes for development of new drugs. The search for new antimalarials is also hampered by the fact that we do not know how most of the current ones work, and we do not know enough about the basic biochemistry of the parasite, including its receptors or enzymes that might act as potential drug targets. However, some molecular targets for antimalarials are known. Among these is the bifunctional enzyme dihydrofolate reductase-thymidylate synthase. Other enzymes in the folate biosynthesis, salvage and utilization pathways are also potential targets. This paper presents a review of work from our group covering identification of enzymes and pathways in folate metabolism of *P. falciparum*, and putting emphasis on the use of parasite dihydrofolate reductase, to search for new antifolate drugs. The latter is made possible through our ability to clone and express the enzyme and their myriad mutants in large amounts from a specially designed synthetic gene.

ENZYMES IN *PLASMODIUM* FOLATE *DE NOVO* SYNTHESIS

Folate metabolism of the malaria parasite, like in bacteria, differs from that of the host to the extent which allows development of drugs with preferential action. Both the pathways for acquisition and utilization of this essential cofactor, and the enzymes catalyzing particular reactions, are different in the parasite and the host. Fig. 1 shows the pathway for formation of parasite folate cofactors as compiled from the work of many groups including ours.[1-6] The parasite has an active *de novo* synthesis pathway, as deduced from the fact that the major folate cofactor of the parasite can be formed *de novo* from guanosine, *p*-aminobenzoate

and glutamate.[2] We identified this major cofactor as methyltetrahydropteroylpentaglutamate,[2] although Hong *et al.*[7] recently found tetraglutamate as the main folate. The discrepancy remains to be explained, but may be related to the use of different parasite strains and different conditions of the experiments.

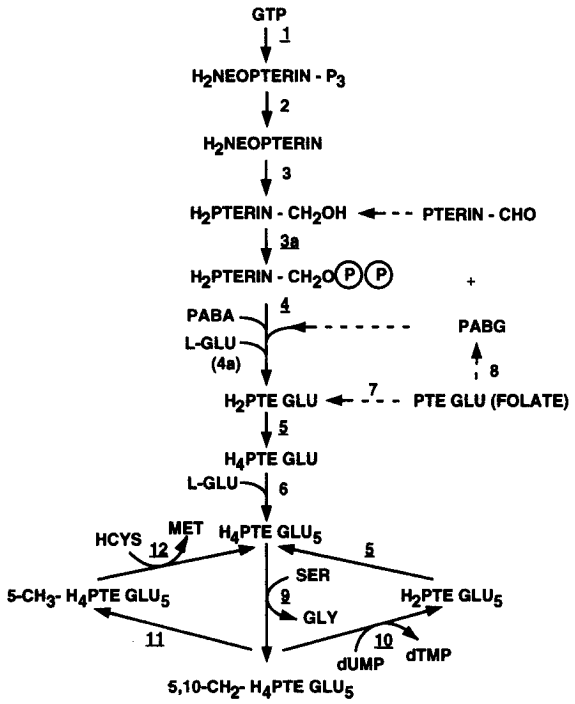

Fig. 1 The *de novo* pathway for synthesis of folate cofactors. The figure also shows one mechanism for folate salvage, and the interconversions in the thymidylate synthesis and methionine synthesis pathways.

The existence of the entire *de novo* pathway starting from guanosine triphosphate (GTP) was also shown by the existence of the first enzyme of this pathway, GTP cyclohydrolase **1**.[3] This enzyme has been partially purified and characterized in *P. falciparum*, *P. knowlesi* and *P. berghei*. *P. falciparum* synthesizes dihydroneopterin triphosphate from GTP, as shown by HPLC using radiolabelled substrate. The next two enzymes in the pathway, dihydroneopterin triphosphate pyrophosphohydrolase **2** and dihydroneopterin aldolase **3**, still remain to be characterized. Recent work[8] has identified the gene coding for dihydrohydroxymethylpterin pyrophosphokinase **3a** adjacent to dihydropteroate synthase **4**, forming a bifunctional protein. Another remaining mystery is that the enzyme dihydrofolate synthase, adding glutamate to dihydropteroate, following the action of dihydropteroate synthase **4a**, has never been detected in *Plasmodium*. It is possible that the parasites use *p*-aminobenzoylglutamate (PABG) as substrate for dihydropteroate synthase, giving rise to dihydrofolate directly instead of dihydropteroate from *p*-aminobenzoate (PABA). The fact that the major folate cofactor in the parasite is a polyglutamate also implies the existence of pteroyl polyglutamate synthase, although this also has not yet been identified.

THE FOLATE SALVAGE PATHWAYS

Our work has also highlighted the importance of the salvage pathway, and has clarified earlier misunderstanding on the sole predominance of the *de novo* pathway. Recent work from our laboratory has shown at least two possibilities of this salvage, one from folate or its degraded moieties[2] and another from 5-methyltetrahydrofolate[5] (Fig. 2). The first pathway[2] was shown by incorporation of exogenous folate into the major folate cofactor, presumably following the action of folate reductase **7**. Alternatively, exogenous folate can be degraded to pterin aldehyde and *p*-aminobenzoylglutamate through the action of folate degrading enzyme(s). Both moieties can then join the metabolic pool as shown in Fig. 1. The second pathway[5] was shown by incorporation of the folate moiety from 5-methyltetrahydrofolate, previously introduced into red cells, into parasite polyglutamylated folate pool, and incorporation of the methyl group into methionine. The possible significance of this second pathway will be explained presently.

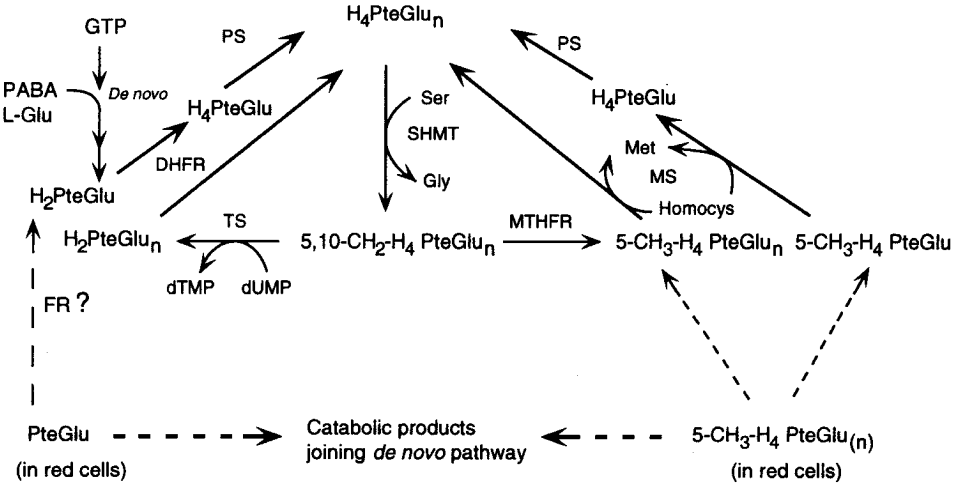

Fig. 2 Interconversions of folate cofactors obtained through either *de novo* synthesis or salvage, including both changes in oxidation levels and polyglutamylation, occur through the adjoining thymidylate synthesis and the methionine synthesis cycles.

In addition to the formation of folate cofactors, two adjacent pathways utilizing the cofactors and sharing a common enzyme also exist in the malaria parasites: the thymidylate synthesis pathway and the methionine synthesis pathway (Fig. 1). The enzyme serine hydroxymethyltransferase (SHMT, **9**) takes the hydroxymethyl group from serine and forms 5,10-methylenetetrahydrofolate or its polyglutamylated derivative, which can then transfer its C_1 moiety in one of the two pathways. Unlike the enzymes of the thymidylate pathway, which provides deoxythymidylate essential for the synthesis of DNA, those of the presumed methionine synthesis pathway had not been characterized until recently. We have identified and characterized 5,10-methylenetetrahydrofolate reductase **11**[5] and methionine synthase **12**[4] from *P. falciparum*. However, although all the three enzymes of this cycle are present with significant specific activities in crude parasite extracts, we could not demonstrate the C_1 transfer from serine to methionine, as might be expected.[5] Therefore, the methionine synthesis cycle must be far less active than the adjoining thymidylate synthesis cycle. However, the significance of the methionine synthesis cycle for the parasite is probably not for production of methionine, which can be obtained exogenously, but for generation of active folate cofactors from more stable precursors. Rather, its significance probably lies in providing the route for folate salvage, and for the parasites to form polyglutamate derivatives of folate.

The formation of pteroylpolyglutamates from monoglutamate precursors implies the presence of polyglutamate synthase in *P. falciparum*, although this has yet to be identified and characterized. This would form tetrahydropteroylpolyglutamates from the monoglutamate obtained from *de novo* synthesis or salvage. Since polyglutamylated derivatives are better substrates for folate conversions than the monoglutamate, and since methyltetrahydropteroylpolyglutamate is the major form of the folate cofactor, the methionine synthesis cycle thereby assumes significance in providing the mechanisms for folate conversions and polyglutamylation in the second pathway for folate salvage (Fig. 2). Likewise, the thymidylate synthesis cycle, in addition to providing dexythymidylate required for parasite DNA synthesis, also serves a role in polyglutamylation of folates obtained from *de novo* synthesis and the first pathway of folate salvage (Fig. 2).

DIHYDROFOLATE REDUCTASE-THYMIDYLATE SYNTHASE

The most important targets in folate metabolism are dihydrofolate reductase-thymidylate synthase (DHFR-TS, **5** and **10**) bifunctional enzymes.[9] These constitute two of the three enzymes in the thymidylate synthesis cycle (**10**, **5** and **9** of Fig. 1). Parasite DHFR is inhibited by such antifolates as pyrimethamine, cycloguanil and trimethoprim. However, resistance usually develops easily against these antifolates. The mechanism for resistance of the parasites to antifolates has been well established as being due to point mutations. This is in contrast with resistance to antifolate drugs in cancer or bacterial infections, which are often due to gene amplification and increased expression of the DHFR. Pyrimethamine resistance, which can

be easily induced, is due to mutation at position 108 from Thr to Asn, causing reduction in inhibitor binding to the enzyme, with additional mutations at positions 59 (Cys to Arg) and 51 (Asn to Ile) contributing to enhancement of resistance.[9-12] The mutation Ala to Val at position 16 resulted in exclusively resistance to cycloguanil. The possible different determinants of resistance to different antifolates carry interesting implications for antifolate chemotherapy, since in bacteria and cancer cells antifolate resistance, due to gene amplification rather than mutation, does not have such drug specificity. It was concluded, for example, that at least three mutations are required for cross resistance to develop, and that a combination of antifolates, say pyrimethamine and proguanil, could delay the on-set of resistance.[12]

In order to develop new drugs from inhibitors against these enzymes, or to study the nature of drug resistance, a large amount of the enzymes is needed. This could not be obtained from conventional preparation from the parasites, since the yields are very low and the purification procedures are tedious. We therefore resorted to cloning and expression of the enzymes from *E. coli*. The cloning of the enzyme was first accomplished by using the parasite gene or its mutants obtained from the parasites in nature.[13] Although this gave significant amounts of the enzymes, the yields were still too low to be of practical use. We reasoned that one reason for inefficient expression of the parasite gene is the fact that the "language" (specified by DNA sequences called codons) used by the parasite is unfamiliar to *E. coli*. One way to overcome this problem is to use the DNA language familiar to *E. coli*, but still leading to the same parasite enzyme. This means that a synthetic gene should be designed and made from chemical reactions, to be used for cloning in the bacterial host. Furthermore, in order to study the binding of the enzyme with potential inhibitors in detail, we would need an efficient means to generate various mutants, both found and not found in nature. A properly designed synthetic gene would allow us to make any number of mutants at any sites, by simply cutting off a small piece of DNA and replacing it with a mutant sequence. Such synthetic gene coding for *P. falciparum* bifunctional enzyme, dihydrofolate reductase-thymidylate synthase (DHFR-TS) has been designed and assembled (Fig. 3).[14] As it turned out, the expression level was not very different from that obtained from natural genes, but another method for cloning was developed which gave rise to a very good yield of the DHFR part of the bifunctional enzyme.[14] Recently, another group has also reported the cloning and expression of the DHFR synthetic gene with an independent design.[15] However, lacking numerous restriction sites designed into the gene, it does not allow facile cassette mutagenesis as our gene.

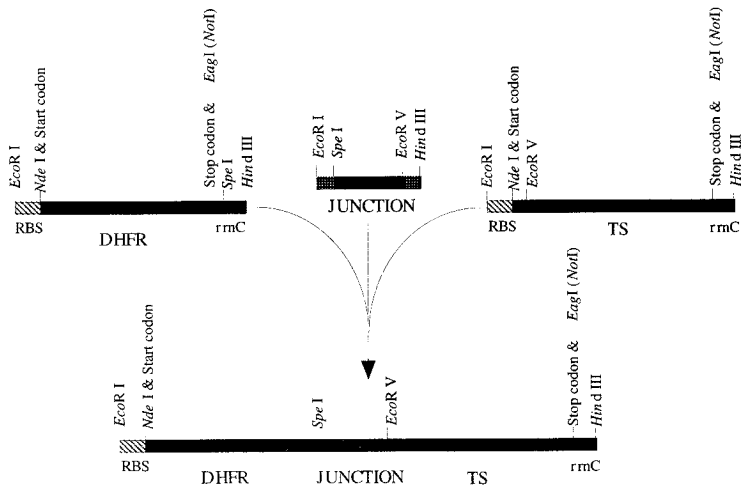

Fig. 3 The design of the synthetic gene for malarial dihydrofolate reductase-thymidylate synthase

With the synthetic gene at hand, and a new method to express large amounts of the DHFR, the way is now open for us to study the properties of both wild-type and mutant enzymes, either earlier found in nature, or yet to be found, or even theoretically not expected to be found because of unlikelihood of natural mutations (for example, mutations at two adjacent sites). This should enable us to understand the mechanisms

of resistance at the molecular level. We have found that mutation at specific sites, earlier known to produce drug-resistant parasites in nature, indeed leads to less binding of DHFR from synthetic genes with pyrimethamine and cycloguanil as expected, hence validating the approach used.[13] Furthermore, from the study of various mutant enzymes, both found and not found in nature (see Table 1), we can show that there is a strong correlation between the binding affinities of pyrimethamine and cycloguanil to mutant enzymes, with the exception of the mutations at position 16 (see Fig. 4).[16] The current hypothesis that there is little cross resistance in general between pyrimethamine and cycloguanil, therefore, has been put in serious doubt. We have also produced all the 20 possible mutant enzymes with mutations at position 108, and from detailed analysis can now understand why the mutant with asparagine in this position is the predominant one in nature for pyrimethamine resistance: the other mutants are either inactive enzymes or still bind well with pyrimethamine.[17] Only the glutamine mutant would have both a good enzyme activity and reduced binding with pyrimethamine, but this mutant requires two successive mutations at adjacent sites, unlikely to occur in nature.

Table 1 List of mutants constructed by cassette mutagenesis of wild-type synthetic *P. falciparum* DHFR domain

Name of construct	Amino acid residue of DHFR domain				
	16	51	59	108	164
Wild-type	Ala	Asn	Cys	Ser	Ile
S108N	Ala	Asn	Cys	***Asn***	Ile
S108T*	Ala	Asn	Cys	***Thr***	Ile
C59R*	Ala	Asn	***Arg***	Ser	Ile
N51I*	Ala	***Ile***	Cys	Ser	Ile
N51I+S108N	Ala	***Ile***	Cys	***Asn***	Ile
C59R+S108N	Ala	Asn	***Arg***	***Asn***	Ile
N51I+C59R+S108N	Ala	***Ile***	***Arg***	***Asn***	Ile
I164L*	Ala	Asn	Cys	Ser	***Leu***
C59R+S108N+I164L*	Ala	Asn	***Arg***	***Asn***	***Leu***
N51I+C59R+S108N+I164L	Ala	***Ile***	***Arg***	***Asn***	***Leu***
A16V*	***Val***	Asn	Cys	Ser	Ile
A16V+S108T	***Val***	Asn	Cys	***Thr***	Ile
A16V+S108N*	***Val***	Asn	Cys	***Asn***	Ile

* Mutants which are not found in nature

From the general correlation between binding affinities to pyrimethamine and the level of parasite drug resistance resulting from point mutations, we can postulate a sequential mutation mechanism for development of resistance. Resistance to pyrimethamine probably first occurs through mutation at position 108, followed by sequential mutations at other sites, giving increasingly resistant parasites, which are also cross resistant against cycloguanil. Unique cycloguanil resistance, however, seems to have occurred through S108T (serine to threonine at 108) and A16V (alanine to valine at 16).

CONCLUSION

Malaria is by far still the largest public health threat of the world, in terms of life-threatening incidences and the total number of people in the endemic areas. This is all the more so because it occurs mostly in developing countries like Thailand, which does not have the financial means nor the public infrastructure to prevent and treat it properly. We chose to attack the problem from the angle of a known

target for antimalarials, parasite dihydrofolate reductase, and the metabolic pathways involving folate biosynthesis and salvage. We hope to obtain new leads to antimalarial chemotherapy, and new knowledge on the biochemistry of this parasite, thereby making a small contribution to the global pool of effort to combat malaria.

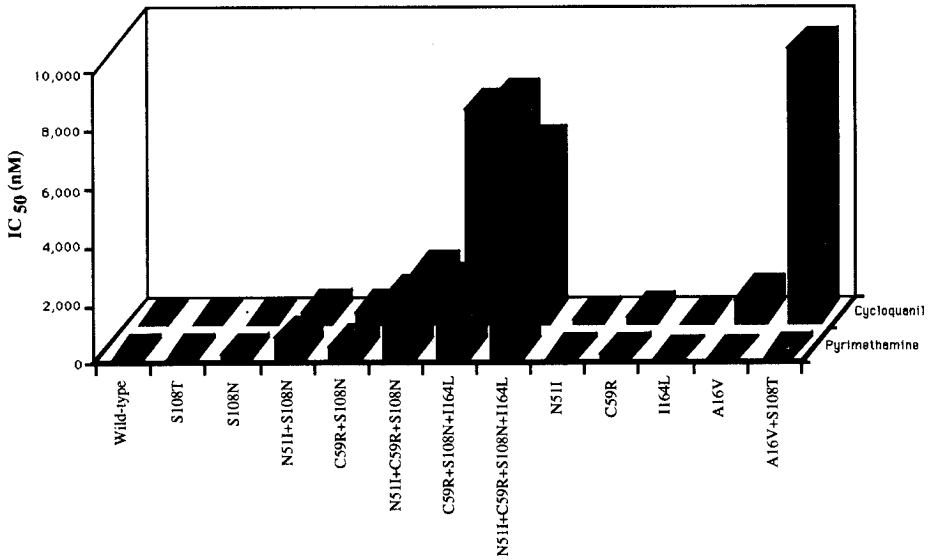

Fig. 4 IC_{50} values for mutants of *P. falciparum* dihydrofolate reductase.

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REFERENCES

1. Wernsdorfer, W. H. and Trigg, P., in ***Malaria. Principles and Practice of Malariology***, **Vol. 2**, Wernsdorfer, W. H. and McGregor, I., eds. (1988), pp. 1569, Churchill-Livingston, Edinburgh.
2. Krungkrai, J., Webster, H. K. and Yuthavong, Y. (1989) ***Mol. Biochem. Parasitol.* 32**, 25.
3. Krungkrai, J., Webster, H. K. and Yuthavong, Y. (1985) ***Mol. Biochem. Parasitol.* 17**, 265.
4. Krungkrai, J., Webster, H. K. and Yuthavong, Y. (1989) ***Parasitol. Res.* 75**, 512.
5. Asawamahasakda, W. and Yuthavong, Y. (1993) ***Parasitology* 107**, 1.
6. Krungkrai, J., Webster, H. K. and Yuthavong, Y. (1990) ***Parasitol. Today* 6**, 388.
7. Hong, Y., Bartlett, M. S., Queener, S., Smith, J. W., Shaw, M. and Meshnick, S. R. (1995) ***FEMS. Microbiol. Lett.* 134**, 251.
8. Triglia, T. and Cowman, A. F. (1994) ***Proc. Nat. Acad. Sci. U.S.A.* 91**, 7149.
9. Hyde, J. E. (1990) ***Pharmac. Ther.* 48**, 45.
10. Peterson, D. S., Walliker, D. and Wellems, T. E. (1988) ***Proc. Natl. Acad. Sci. U.S.A.* 85**, 9114.
11. Cowman, A. F., Morry, M. J., Biggs, B. A., Cross, G. A. and Foote, S. J. (1988) ***Proc. Natl. Acad. Sci. U.S.A.* 85**, 9109.
12. Foote, S. J. and Cowman, A. F. (1994) ***Acta Tropica* 56**, 157.
13. Sirawaraporn, W., Sirawaraporn, R., Cowman, A. F., Yuthavong, Y. and Santi, D. V. (1990) ***Biochemistry* 229**, 10779.
14. Sirawaraporn, W., Prapunwattana, P., Sirawaraporn, R., Yuthavong, Y. and Santi, D. V. (1993) ***Biochemistry* 268**, 21637.
15. Sano, G., Morimatsu, K. and Horii, T. (1994) ***Mol. Biochem. Parasitol.* 63**, 265.
16. Sathitkul, T. (1995) ***M.Sc. Thesis***, Mahidol University.
17. Yongkiettrakul, S. (1996) ***M.Sc. Thesis***, Mahidol University.