บทฟื้นฟูวิชาการ

Biochemistry of Malaria I : folate and cobalamin metabolism and mechanism of pyrimethamine resistance in Plasmodium falciparum.

Jerapan Krungkrai*

Krungkrai J, Biochemistry of malaria I : folate and cobalamin metabolism and mechanism of pyrimethamine resistance in Plasmodium falciparum. Chula Med J 1991 Feb; 35(2):105-111

Malaria is the most prevalent and most devastating parasitic disease of the tropics, despite considerable research and control efforts. At present there is little understanding of the biochemistry of malaria, hence the drug design based on a rational approach is impossible. Here, we discuss the work done with Plasmodium falciparum involving folate and cobalamin metabolism and the implications of this work for the new knowledge of the mechanism of antifolate drug resistance. In addition, a new potential target on cobalamin metabolism is considered.

Reprint request : Krungkrai J, Department of Biochemistry, Faculty of Medicine. Chulalongkorn University, Bangkok 10330, Thailand.
Received for publication. December 11, 1990.

* Department of Biochemistry, Faculty of Medicine, Chulalongkorn University.

จิระพันธ์ กรึงไกร. ชีวเคมีของเชื้อมาลาเรีย I : เมตาบอลิสม์ของวิตามินโฟเลตและโคบาลามีนและกลไกการดื้อยาไพรินเมทธามีน ของเชื้อ พลาสโมเดียม ฟัลซิพารัม. จุฬาลงกรณ์เวชสาร 2534 กุมภาพันธ์; 35(2): 105-111

มาลาเรียเป็นโรคเขตร้อนที่สำคัญที่สุดโดยจะพบทั่วไปในประเทศแถบร้อน ทั้ง ๆ ที่ได้มีการศึกษาและการป้องกันโรคนี้อย่างต่อเนื่อง อาจเป็นผลมาจากมีการแพร่กระจายของเชื้อมาลาเรียที่ดื้อต่อยาที่ได้ใช้รักษา และของยุงพาหะที่ดื้อต่อยาฆ่าแมลง ในปัจจุบันความรู้ที่เกี่ยวกับชีวเคมีของเชื้อมีค่อนข้างน้อย ทำให้การพัฒนายารักษามาลาเรียตัวใหม่ โดยอาศัยความเข้าใจของตำแหน่งเป้าหมายใหม่เป็นไปได้ยาก ในบทความนี้ผู้เขียนได้ทบทวนวารสารที่เกี่ยวข้องกับเชื้อมาลาเรียชนิด Plasmodium falciparum ในส่วนเมตาบอลิสม์ของวิตะมินโฟเลตและโคบาลามีน รวมทั้งการนำไปใช้อธิบายถึงกลไกการดื้อยาไพริเมทธามีนที่มีผลต่อเมตาบอลิสม์ของโฟเลต นอกจากนี้ผู้เขียนได้เสนอเมตาบอลิสม์ของโคบาลามีนอาจเป็นตำแหน่งเป้าหมายใหม่ในการพัฒนายารักษามาลาเรียต่อไป

Malaria is caused by a protozoan parasite of the genus *Plasmodium*, of which 4 species are known to infect humans: *P. falciparum, P. vivax, P. ovale* and *P. malariae*. The transmission of malaria is indigenous in 102 countries and about 2700 million people live in areas endemic for the disease. Over 6 million cases of malaria are treated annually and in Africa alone one million infant deaths can be attributed directly to malaria. Although accurate global evaluation of the frequency of clinical malaria is made difficult by underdetection or under-reporting, the total incidence may be in the order of 100 million cases a year. Malaria remains, despite considerable research and control efforts, the most prevalent and most devastating parasitic disease of the tropics. In the period since 1960, chloroquine-resistant *P. falciparum* has now been confirmed throughout Africa, Asia and South America and is spreading rapidly. The problem of malaria control is well understood by all private, national and international organizations concerned with human health. At present there are two recognizable approaches to the problem; one based on empirical drug screening and the other based on vaccines. While both of these approaches may be valid, they have so far achieved little success. In fact it is now widely conceded that it is unlikely that either approach will make a significant impact on the control of malaria before the disease reaches epidemic proportions.

There is currently very little understanding of the biochemistry of malaria; consequently, it is impossible to design drugs on any kind of rational basis. In this review, wer summarize the work done with *P. falciparum* and other *Plasmodium* spp. involving folate metabolism with respect to metabolic pathway of folate biosynthesis and folate-dependent reactions, including the cobalamin-dependent activity of methionine synthase. We also discuss the implications of this work for understanding the mechanism of antifolate drug resistance and the importance of cloning the dihydrofolate reductase gene. In addition, the role of cobalamin in *P. falciparum* will be considered. Interference of cobalamin utilization may represent a new target for combating the parasite.

Malaria life cycle and chemotherapeutic targets

The malaria infection is initiated when the female anopheline mosquito takes a blood meal from a human host. If the mosquito is infected, sporozoites will be present in its saliva, an inoculum of which will be injected into the host during feeding. Sporozoites disappear from the blood within 20 minutes and enter hepatocytes. In the hepatocyte the parasite undergoes a phase of asexual reproduction or schizogony (preerythrocytic cycle). This tissue stage of infection will eventually produce serveral thousand merozoites which rupture the hepatocyte and enter the systemic circulation. In *P. vivax*, a proportion of sporozoites remains dormant after entering a hepatocyte (called hypnozoite). These hypnozoites undergo development months later and are responsible for the clinical relapses associated with some forms of malaria. About 55-60% of malaria infection in Thailand is *P. falciparum*, the rest is *P. vivax*.

The released merozoites penetrate erythrocytes (erythrocytic phase) and form trophozoites. These trophozoites undergo rapid growth followed by a stage of asexual schizogony. The mature schizont ruptures, releasing several merozoites capable of infecting new erythrocytes. It is this stage of the infection which is of clinical importance. Some trophozoites differentiate into gametocytes which are infective to the mosquito vector. These are several points in the malaria life cycle where therapeutic intervention could be useful. A number of antimalarial drugs including the antifolate, the sesquiterpene lactones (artemisinine, artesunate and artemether), the quinoline methanols (quinine, quinidine and mefloquine), the recently developed phenanthrenemethanol halofantrine and the 4-aminoquinolines (amodiaquine and chloroquine) have been used as schizontocides against the erythrocytic stages of malaria. The 8-aminoquinoline primaquine is the drug of choic against the preerythrocytic stages of malaria. It also possesses potent gametocytocidal action.

Folate and cobalamin biochemistry

Folate coenzymes serve as acceptors or donors of one-carbon units in a variety of reactions involved in purine, pyrimidine and amino acid metabolism and in the initiation of protein synthesis.[1,2] The coenzyme form of the vitamin are the tetrahydro derivatives which can accept one-carbon units. A reaction catalyzed by enzyme methionine synthase is known to require cobalamin (vitamin B_{12}) as cofactor. The intracellular folate coenzymes are present predominantly as polyglutamate derivatives with gamma linkage of glutamyl residues. The folylpolyglutamates (or pteroylpolyglutamates) are better substrates than folate (monoglutamate form) for many folate-dependent enzymes so far examined.[3] Most mammalian cells utilize exogenous folate as the monoglutamate of 5-methyltetrahydrofolate which is the circulating form in the plasma.

Folate biosynthetic pathway in P. falciparum

In contrast to the host system, malaria parasites have the ability to synthesize their folates. The folate metabolism in malaria parasites especially in *P.*

falciparum is not well understood,[4-6] but one potential target for chemotherapeutic attack in combating human malaria is dihydrofolate reductase, an important enzyme of folate biosynthesis in the parasite. From the chemistry of folate molecule, it consists of 3 parts : heterocyclic pteridine, p-aminobenzoate (PABA) and L-glutamate. PABA is known to be an essential growth factor for malaria growth which is susceptible to sulfonamides.[4] Heterocyclic pteridines have been shown to be synthesized by the parasite from guanosine 5′ triphosphate (GTP) by the enzyme GTP cyclohydrolase.[7] This extremely labile enzyme has been partially purified and characterized in *P. falciparum, P. knowlesi and P. berghei.* By using radiolabelled either guanosine or GTP, it was demonstrated that *P. falciparum* grown *in vitro*[8] synthesizes dihydroneopterin and neopterin from these labelled precursors.[9] The synthesis of these pteridines by the parasites is strongly inhibited by 7-methylguanosine, a strong competitive inhibitor of malarial GTP cyclohydrolase that has antimalarial activity *in vitro* against *P. falciparum.*[7] The next two enzymes of pteridine synthesis in malaria, dihydroneopterin triphosphate pyrophosphohydrolase and dihydroneopterin aldolase, remain to be characterized. In a series of experiments using metabolic labelling techniques the complete pathway of *de novo* biosynthesis of folate has been recently discovered.[9] *P. falciparum* synthesizes folates *de novo* from these precursors GTP, PABA and L-glutamate, forming 5-methyltetrahydropteroylpentaglutamate (5-CH_3-H_4PteGlu$_5$), the major end product, as shown in Fig. 1

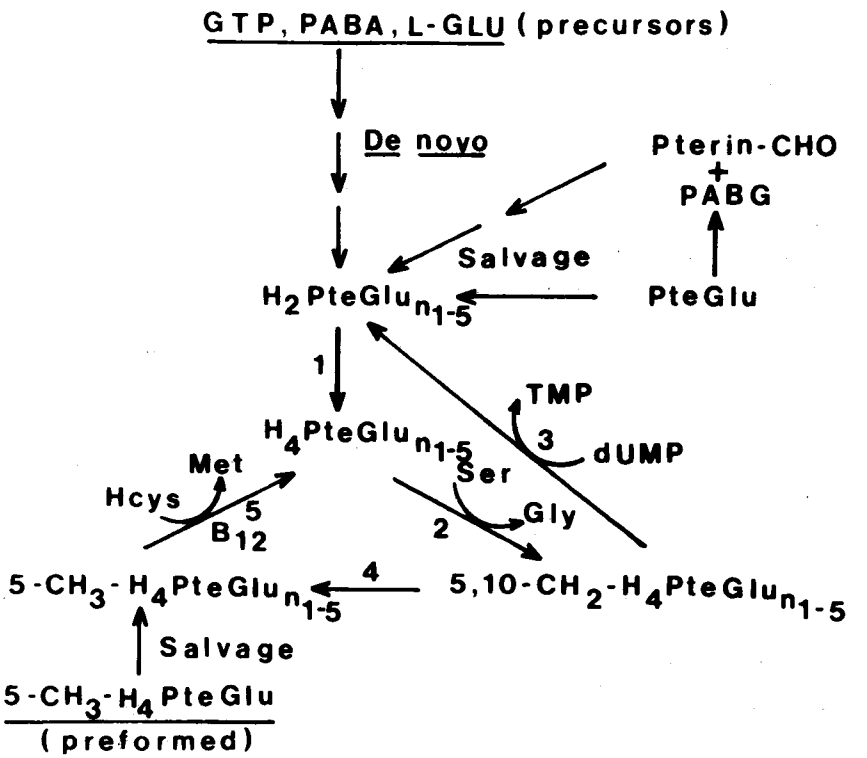

Figure 1. De novo biosynthesis and salvage of folate in *P. falciparum.* Enzymes: 1, dihydrofolate reductase (DHFR); 2, serine hydroxymethyltransferase (SHMT); 3, thymidylate synthase (TS); 4, methylene-tetrahydrofolate reductase (MTHFR); 5, methionine synthase.

Folate salvage pathway in P. falciparum

It has been suggested that malaria parasite has the ability to utilize exogenous folate as both intact and degraded forms.[10-12] We have confirmed this observation with the finding that radiolabelled folate is incorporated into 5-methyl-H_4PteGlu$_5$, the end product of folate *de novo* synthesis and by showing that its incorporation is sensitive to sulfadoxine (pterin-aldehyde and p-aminobenzoylglutamate, PABG, as degraded folate) and pyrimethamine (intact molecule) as shown in Fig. 1.

The utilization of host erythrocyte 5-CH_3-H_4 PteGlu$_5$, the major intracellular folate, is possible but this is still unproven. However, 5-methyl-tetrahydrofolate (5-CH_3-H_4PteGlu) could be obtained from host plasma

by the parasite. We have recently shown the presence of methionine synthase in *P. falciparum.*[13] This enzyme may be responsible for salvaging 5-CH_3-H_4PteGlu from the host plasma. This may also be an alternative route for obtaining the folate cofactors by the parasite (see Fig. 1)

Tetrahydrofolate and cobalamin metabolism in malaria

In protozoa, enzyme dihydrofolate reductase (DHFR), catalyzing the conversion of dihydrofolate (H_2PteGlu) to tetrahydrofolate (H_4PteGlu), and thymidylate synthase (TS), catalyzing the conversion of deoxyuridine 5'-monophosphate (dUMP) to thymidine 5'-monophosphate (TMP), enzyme number 1 and 3 of Fig. 1, exists as a bifunctional protein.[14] The enzyme has been well characterized and their genes cloned. Serine hydroxymethyltransferase (SHMT) catalyzes conversion of H_4PteGlu to 5,10-methylene-tetrahydrofolate (5,10-CH_2-H_4PteGlu), enzyme number 2 of Fig. 1, to complete the thymidylates synthesis cycle[15] :

$$H_2PteGlu \xrightarrow{DHFR} H_4PteGlu \xrightarrow{SHMT} 5,10\text{-}CH_2\text{-}H_4PteGlu \xrightarrow{TS} H_2PteGlu.$$

It was recently discovered that the SHMT enzyme isolated from pyrimethamine-sensitive and resistant clones of *P. chabaudi* had similar kinetic properties.[16]

It is also suggested that a methionine synthesis cycle exists in the malarial parasite[4,6] :

$$H_4PteGlu \xrightarrow{SHMT} 5,10\text{-}CH_2\text{-}H_4PteGlu \xrightarrow{MTHFR} 5\text{-}CH_3H_4PteGlu \xrightarrow{MS} H_4PteGlu$$

these reactions are catalyzed by SHMT, methylene-tetrahydrofolate reductase (MTHFR) and methionine synthase, respectively. We have focused our attention on methionine synthase because the enzyme is known to require vitamin B_{12} (cobalamin) as cofactor and there is a very little understanding on the relationship between folate and cobalamin in malaria. The methionine synthase is recently purified and characterized from *P. falciparum.*[13] The malarial enzyme contained tightly bound cobalamin, which is inactivated both *in vitro* and *in vivo* by nitrous oxide (N_2O).

The growth of malaria parasites from young ring-stages to schizonts in N_2O-treated cultures in the presence of 17% O_2, 3% CO_2 and balanced with N_2 atmosphere is markedly inhibited (Fig. 2). Our results indicate for the first time the importance of cobalamin for parasite

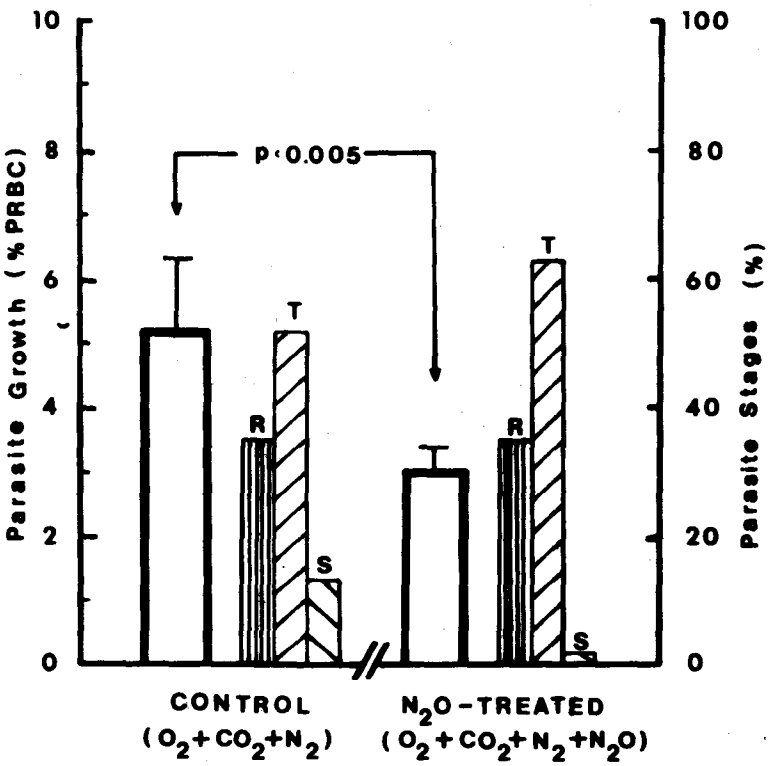

Figure 2. Effect of N_2O on *P. falciparum* growth in *in vitro.* The parasite growth was measured using morphological end point at 48 h in the presence of N_2O, compared to that control culture with a gas mixture of O_2, CO_2 and N_2. Open bars, percentage of parasitemia (% PRBC); R,T and S bars, ring, trophozoite, and schizont stages, respectively.

growth *in vitro*. Cobalamin metabolism may offer a potential target for the development of new antimalarial drugs.

Mechanism of pyrimethamine resistance in P. falciparum

The increase of antimalarial resistance partly reflects a lack of understanding of drug resistance mechanism. The antifolate drug pyrimethamine is used as an effective antimalarial agent, acting against parasite by inhibiting enzyme dihydrofolate reductase or DHFR.[17] Naturally occurring resistance to pyrimethamine has been described, and resistant mutants have been characterized and isolated by mutagenesis and drug selection.[18-23]

Resistance to pyrimethamine could result from a number of mechanisms through which changes in drug susceptibility may occur. These include (1) Point mutations of the DHFR-TS gene on chromosome 4, resulting in an altered enzyme having the amino acid changes at positions 223 (serine to phenylalanine), 108 (threonine to asparagine), 59 (cysteine to arginine) or 16 (valine to alanine).[20-25] Recent evidence on chromosomal rearrangement in pyrimethamine-resistant parasites[26-27] suggest that point mutation(s) and chromosomal change are closely associated with the pyrimethamine resistance by the parasite. The use of pyrimethamine-resistant *P. falciparum* obtained from endemic areas suggests that the drug resistance may be involved in the decrease affinity of the drug binding to the mutant enzyme.[20,21,24,28] (2) Duplication and amplification of the DHFR-TS gene, increasing the amount of normal DHFR-TS[29], defective DHFR-TS.[27,30] (3) Reduction of the drug uptake. The reduction of antifolate drug uptake by *Leishmania* has been involved in one of the mechanism of the resistance to the drug.[31] However, although this occurs in a resistant mutant[18], it is not found in a resistant *P. falciparum*[32]. (4) *Metabolic alterations*. An example might be increased salvage of 5-methyl-tetrahydrofolate which can be converted to tetrahydrofolate through methionine synthase, bypassing the dihydrofolate reductase step.

Of all these possibilities, mechanism (1) has been clearly shown for *P. falciparum*, while other mechanisms still remain to be clearly proven. Furthermore, it should be noted that the mutant enzyme in mechanism (1) also decreased the binding to substrate dihydrofolate, resulting in altered folate metabolism.[24] The increase of folate utilization and synthesis by the pyrimethamine-resistant parasite has been observed in response to these changes.[9] Comparative studies on the specific activities of enzymes involving one-carbon transfer reaction between the drug-sensitive and resistant parasites might yield further insights into the resistance mechanism.

Acknowledgement

This work was supported in part by UNDP/World Bank/WHO Special programme for Research and Training in Tropical Diseases.

References

1. Stokstad ELR, Koch J. Folic acid metabolism. Physiol Rev 1967 Jan; 47(1) : 83-116
2. Brown GM, Williamson JM. Biosynthesis of riboflavin, folic acid, thiamine, and pantothenic acid. Adv Enzymol 1982; 53 : 345-81
3. Matthews RG, Ghose C, Green JM, Matthews KD, Dunlap RB. Folylpolyglutamates as substrates and inhibitors of folate-dependent enzymes. Adv Enzyme Reg 1987; 26 : 157-71
4. Ferone R. Folate metabolism in malaria. Bull WHO 1977 Mar; 55 (1) : 291-7
5. Sherman IW. Biochemistry of Plasmodium (malaria parasites). Microbiol Rev 1979 Dec; 43(4) : 453-95
6. Sherman IW. metabolism. In : Peters W, Richards WHG, eds. Antimalarial Drugs I. Springer : Berlin Heidelberg, 1984. 31-81
7. Krungkrai J, Yuthavong Y, Webster HK. Guanosine triphosphate cyclohydrolase in Plasmodium falciparum and other Plasmodium species. Mol Bicochem Parasitol 1985 Dec; 17(3) : 265-76
8. Trager W, Jensen JB. Human malaria parasites in continuous culture. Science 1976 Aug; 193(4254) : 673-5
9. Krungkrai J, Webster HK, Yuthavong Y. De novo and salvage biosynthesis of pteroylpentaglutamates in the human malaria parasite, Plasmodium falciparum. Mol Biochem Parasitol 1989 Jan; 32(1) : 25-38
10. Watkins WM, Sixsmith DG, Chulay JD, Spencer HC. Antagonism of sulfadoxine and pyrimethamine antimalarial activity in vitro by p-aminobenzoic acid, p-aminobenzoylglutamic acid and folic acid. Mol Biochem Parasitol 1985 Jan-Mar; 14(1) : 55-61

11. Milhous WK, Weatherly NF, Bowdre JK, Desjardins RE. In vitro activities of and mechanisms of resistance to antifol antimalarial drugs. Antimicrob Agents Chemother 1985 Apr; 27(4) : 525-30
12. Dieckmann A, Jung A. Mechanism of sulfadoxine resistance in Plasmodium falciparum. Mol Biochem Parasitol 1986 May; 19(2) : 143-7
13. Krungkrai J, Webster HK, Yuthavong Y. Characterization of cobalamindependent methionine synthase purified from thehuman malarial parasite, Plasmodium falciparum. Parasitol Res 1989 Sep; 75(4) : 512-7
14. Garrett CE, Coderre JA, Meek TD, Garvey EP, Claman DM, Beverley SM, Santi DV. A bifunctional thymidylate synthase-dihydrofolate reductase in protozoa. Mol Biochem Parasitol 1984 Apr; 11(1) : 257-65
15. Platzer EG. Metabolism of tetrahydrofolate in Plasmodium lophurae and ducking erythrocytes. Trans N Y Acad Sci 1972 Mar; 34(3) : 200-8
16. Ruenwongsa P, Luanvararat M, O' Sullivan WJ. Serine hydroxymethyltransferase from pyrimethamine-sensitive and- resistant strains of Plasmodium chabaudi. Mol Biochem Parasitol 1989 Mar; 33(3) : 265-72
17. Ferone R, Burchall JJ, Hitchings GH. Plasmodium berghei dihydrofolate reductase. Isolation, properties, and inhibition by antifolates. Mol Pharmacol 1969 Jun; 5(1) : 49-59
18. Banyal HS, Inselburg J. Plasmodium falciparum : induction, selection, and characterization of pyrimethamine-resistant mutants. Exp Parasitol 1986 Aug; 62(1) : 61-70
19. Bzik DJ, Li W, Horii T, Inselburg J. Molecular cloning and sequence analysis of the Plasmodium falciparum dihydrofolate reductase thymidylate synthase gene. Proc Natl Acad Sci USA 1987 Dec; 84(23) : 8360-4
20. Peterson SD, Walliker D, Wellems TE. Evidence that a point mutation of dihydrofolate reductase-thymidylate synthase confers resistance to pyrimethamine in falciparum malaria. Proc Natl Acad Sci USA 1988 Dec; 85(23) : 9114-8
21. Cowman AF, Morry MJ, Biggs BA, Cross GAM, Foote SJ. Amino acid changes linked to pyrimethamine resistance in the dihydrofolate reductase-thymidylate synthase gene of Plasmodium falciparum. Proc Natl Acad Sci USA 1988 Dec; 85(23) : 9109-13
22. Inselburg J, Bzik DJ, Li W. Plasmodium falciparum : three amino acid changes in the dihydrofolate reductase of a pyrimethamine resistant mutant. Exp Parasitol 1988 Jun; 67(3) : 361-3
23. Zolg JW, Plitt JR, Chen G, Palmer S. Point mutations in the dihydrofolate reductase-thymidylate synthase gene as the molecular basis for pyrimethamine resistance in Plasmodium falciparum. Mol Biochem Parasitol 1989 Oct; 36(3) : 253-62
24. Snewin VA, England SM, Sims PFG, Hyde JE. Characterisation of the dihydrofolate reductase-thymidylate synthetase gene from human malaria parasites highly resistant to pyrimethamine. Gene 1989 Mar; 76(1) : 41-52
25. Tanaka M, Gu HM, Bzik DJ, Li WB, Inselburg J. Dihydrofolate reductase mutations and chromosomal changes associated with pyrimethamine resistance of Plasmodium falciparum. Mol Biochem Parasitol 1990 Feb; 39(1) : 127-34
26. Cowman AF, Lew A. Chromosomal rearrangements and point mutations in the DHFR-TS gene of Plasmodium chabaudi under antifolate selection. Mol Biochem Parasitol 1990 Aug; 42(1) : 21-30
27. Tanaka M, Gu HM, Bzik DJ, Inselburg J. Mutant dihydrofolate reductase-thymidylate synthase genes in pyrimethamine-resistant Plasmodium falciparum with polymorphic chramosomal duplications. Mol Biochem Parasitol 1990 Aug; 42(1) : 83-92
28. Walter Rd. Altered dihydrofolate reductase in pyrimethamine resistance in Plasmodium falciparum. Mol Biochem Parasitol 1986 Apr; 19(1) : 61-6
29. Kan SC, Siddiqui WA. Comparatives on dihydrofolate reductases from Plasmodium falciparum and Aotus trivirgatus. J Protozool 1979 Nov; 26(4) : 660-4
30. Inselburg J, Bzik DJ, Horii T. Pyrimethamine resistant Plasmodium falciparum : overproduction of dihydrofolate reductase by a gene duplication. Mol Biochem Parasitol 1987 Nov; 26(1,2) : 121-34
31. Ellenberger TE, Beverley SM. Reductions in methotrexate and folate influx in methotrexate-resistant lines of Leishamania major are independent of R or H region amplification. J Biol Chem 1987 Oct; 262(23) : 13501-7
32. Dieckmann A, Jung A. The mechanism of pyrimethamine resistance in Plasmodium falciparum. Parasitology 1986 Oct; 93(2) : 275-8