

Biochemistry of malaria II : metabolism of nucleic acids

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*Malaria is a major health problem in tropical parts of the world. With the emergence of drug-resistant strains of **Plasmodium falciparum** a rational biochemical approach to anti-malarial chemotherapy has become more desirable. As essential components of development and replication, nucleic acid precursors are one logical target for non-empirical antimalarial agents. In this paper, we discuss the metabolism of nucleic acids, including both purine and pyrimidine synthesis with special reference to our own work, in malarial parasites and their relation to host mammalian cells. Some key differences of these pathways between the host and malarial parasites are frequently described as a basis for possible chemotherapeutic targets and the development of antimalarial drugs. Based on the catalytic mechanism of enzyme dihydroorotate dehydrogenase action, we have designed and synthesized a quinone analog as a mechanism-based inhibitor. Quinone had moderate antimalarial activity against **P. falciparum** *in vitro* and **P. berghei** *in vivo*.*

Key words : *Malaria, Biochemistry, Nucleic acids.*

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โรคมาลาเรียเป็นปัญหาทางสุขภาพที่สำคัญในประเทศแถบร้อน จากผลที่มีการระบาดอย่างรวดเร็วของสายพันธุ์ที่ดื้อต่อยาที่ใช้รักษาอยู่ของเชื้อพลาสโมเดียมฟัลซิพารัม จึงได้นำวิธีการทางชีวเคมีมาใช้ในการพัฒนารักษามาลาเรียตัวใหม่ๆ กรดนิวคลีอิกและสารตั้งต้นมีความจำเป็นในการเจริญเติบโตและการเพิ่มของจำนวนของเชื้อมาลาเรียจึงเป็นตำแหน่งเป้าหมายที่จะพัฒนารักษามาลาเรียได้ สำหรับบทความนี้จะกล่าวถึงเมตาบอลิซึมของกรดนิวคลีอิก และการสังเคราะห์เบสเพียวรีนและเบสไพริมิดีนที่เป็นสารตั้งต้นของการสังเคราะห์กรดนิวคลีอิกในเชื้อมาลาเรียและเซลล์เจ้าบ้าน โดยเน้นถึงงานวิจัยของผู้เขียนที่ได้ทำไปแล้ว และงานวิจัยของผู้อื่นที่เกี่ยวข้อง ความแตกต่างที่สำคัญในเมตาบอลิซึมของกรดนิวคลีอิกระหว่างเชื้อมาลาเรียและเซลล์เจ้าบ้านที่ค้นพบนี้ จะเป็นตำแหน่งเป้าหมายทางเคมีบำบัดและการพัฒนารักษามาลาเรีย ในบทความยังได้รายงานการค้นพบกลไกการทำงานของเอ็นไซม์ในการสังเคราะห์เบสไพริมิดีน อาทิเช่น เอ็นไซม์ไดไฮโดรออโรเทตดีไฮโดรจีเนส และการพัฒนาและการสังเคราะห์สารที่มีโครงสร้างคล้ายควิโนโนนที่เป็นตัวยับยั้งการทำงานของเอ็นไซม์ตัวนี้ในเชื้อมาลาเรีย ตลอดจนการทดสอบฤทธิ์ต้านเชื้อมาลาเรีย ที่พบว่า มีผลต้านเชื้อได้ระดับปานกลางต่อเชื้อพลาสโมเดียมฟัลซิพารัมในห้องทดลอง และต่อเชื้อพลาสโมเดียมเบอกส์ไอในหนูถีบจักร

Based on an estimation of the World Health Organization, perhaps 110 million clinical cases of malaria occur annually and approximately 1 million infected people die a year.⁽¹⁾ Since the severity of the illness is markedly influenced by the species of *Plasmodium* causing human malaria: *Plasmodium vivax*, *P. ovale* and *P. malariae* rarely produce life-threatening diseases, whereas *P. falciparum* frequently causes severe disease. The presence of drug-resistant *P. falciparum* has made some antimalarial drugs unusable (e.g. chloroquine) in South East Asia and South America. Many new antimalarial drugs are being developed and some are being studied at the pre-clinical and clinical stages.⁽¹⁾ Since these new drugs have been designed on an empirical basis, the mechanism of action of these new drugs remains to be identified. It is now possible to develop new antimalarial drugs rationally on the basis of accumulated knowledge of the biochemical processes of the parasite which can be used to define target metabolites and enzymes.

The complexity of the malaria life cycle has enabled most studies to illustrate the interesting biochemical and physiological processes of malarial parasites only in the intra-erythrocytic stages of *P. falciparum*. There were first successfully cultivated *in vitro* in 1976 by Professor Bill Trager of Rockefeller University.⁽²⁾ In the red cell, the intra-cellular parasite is dependent on the host cell for certain basic nutrients (e.g. glucose, ribose), metabolites (e.g. hypoxanthine, folate) and enzymes (e.g. superoxide dismutase). The infected red cell is usually more permeable to nutrients, cations as Ca^{2+} , and other substances, including toxic substances (e.g. antimalarial drugs).⁽³⁾ The knowledge of host cell-parasite relationship is now progressing well, e.g., it has been found that exogenous macromolecules can be directly accessed through a parasitophorous duct of malarial parasites.⁽⁴⁾

The basic features of the biology of the malarial infection and the major principles and objectives of its treatment was recently reviewed in the journal.⁽⁵⁾ At present although there is little understanding of the biochemistry of malaria, recent knowledge concerning the advantages of

the past experience and new technology has prompted us to discuss the metabolism nucleic acids, including both purine and pyrimidine synthesis, for a review of the biochemistry of malaria; however, we emphasize only the erythrocytic stage of the malaria parasite.

In the first part of this communication, we describe the characteristics of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) molecules of the parasite which are different from those of the host cell; in the second part, we present purine and pyrimidine metabolism in the normal red cell as the basis for our discussion of the changes which occur following malaria parasite infection; and in the third part we consider purine metabolism in the malaria parasite and its relationship to the host red cell and also mention about possible chemotherapeutic targets and newly developed antimalarial agents.

Characteristics of nucleic acids in malarial parasites

Malarial parasites contain DNA, RNA and DNA-bound histones. An intra-erythrocytic ring-stage trophozoite contains about 10^{-13} g of DNA.⁽⁶⁾ The genome of the parasite contains four major bases: of adenine(A), cytosine(C), guanine(G), and thymine(T), shows methylation of cytosine only. It comprises $\sim 3 \times 10^7$ base-pairs (bp) of exceptionally A+T-rich (82%) sequence and extremely low G+C content (18%), which is the lowest reported for a living organism. These contents of the bases are also varied by species of *Plasmodium*, as shown in Table 1. Early studies have shown that primate malarial parasites contained 37% G+C,⁽⁶⁾ later studies have claimed that the content of 37% G+C was contaminated by host white cell.⁽⁷⁾ Analyses of the base composition of malarial parasite DNA, indicates *P. falciparum* is more closely related to rodent (*P. berghei*, *P. chabaudi*, *P. vinckei*, *P. yoelii*) and avian (*P. lophurae*) malarial parasites than to primate (*P. vivax*, *P. malariae*, *P. knowlesi*, *P. cynomolgi*, *P. fragile*) malarial parasites. It is generally accepted that the G+C content of host mammalian cells is $\sim 37\%$, which differs greatly from that of the malarial parasites.

Table 1. The G+C content of various malarial DNA. These values are estimated by the density at which they banded in Hoechst dye cesium chloride gradients. Symbols: +, present; -, absent.

Species	Host	G+C content	
		18%	30%
<i>P.falciparum</i>	Man	+	-
<i>P.berghei</i>	Rodent	+	-
<i>P.lophurae</i>	Bird	+	-
<i>P.knowlesi</i>	Monkey	-	+
<i>P.fragile</i>	Monkey	-	+
<i>P.cynomolgi</i>	Monkey	+	+
<i>P.vivax</i>	Man	+	+

The RNA of the malarial parasite has been little studied; however, the RNA content of malarial parasite is estimated to be five times greater than the DNA content. Most of the malarial RNA is localized in abundant cytoplasmic ribosomes which are present in the intra-erythrocytic parasites. The ribosomal RNA (rRNA) of parasites is typically protozoan in its sedimentation values of 25S and 15S for 60S and 40S ribosomal subunit, respectively, while rRNAs of the mammalian cells are 28S and 18S. The base composition of the rRNAs from the parasites are found to be have a 35% G+C content, whereas that of rRNAs from the host cells have a 64% G+C content. The pattern of rRNA synthesis and the existence of 45S, 37.2S, and 31.7S RNA species suggest that, in malarial parasites, the processing of rRNA may be typically eucaryotic. The characterization of messenger RNA (mRNA) appears to be like that of other eucaryotes in its size range and average chain length of about 1.2 kilobases (kb). The 3'-poly(A) content and average length resemble more closely those of mRNA from higher eucaryotes rather than the shorter chains of lower eucaryotes. Very little is known about transfer RNA (tRNA) in malaria parasites; however, the proportions of rRNA, tRNA and mRNA from *P.falciparum* (82:13:5) are very similar to those found in other organisms.

The malaria parasite is haploid for most of its life-cycle, with zygote formation and meiosis occurring during the mosquito phase of development. as revealed by pulsed-field gradient electrophoresis, *P.falciparum* contains

14 chromosomes which are strikingly polymorphic in size ranging from 600 kb to approximately 3000 kb. These polymorphisms are very useful markers for typing isolates of *P.falciparum* obtained from endemic areas. The mechanisms of these variations have been found to be polymorphisms both in length and in sequence of the subtelomeric regions which are located at the ends of most parasite chromosomes. These are the result of aberrant DNA rearrangements during mitosis. The rearrangements seem to have no specific function and also occur in the DNA of many other eucaryotes.⁽⁸⁾ A number of genes encoding malarial antigens currently viewed as candidate vaccine molecules have been assigned to different chromosomes, e.g., chromosome 1 (ring-infected erythrocytic surface antigen, RESA), chromosome 3 (circumsporozoite antigen, CSP), chromosome 4 (dihydrofolate reductase), chromosome 7 (S antigen), and chromosome 14 (thrombospondin-related anonymous protein).

The intra-erythrocytic stage of *P.falciparum* (namely ring-stage and growing trophozoites, immature and mature schizonts) replicates itself 10-20 times during the last 4-6 hours (mature schizont stage) of its 48-hour life-cycle. If an average malarial parasite produces about 16 merozoite nuclei every 48 hours, the parasite doubling time is approximately six hours. An important consideration would be: what are the syntheses for the nucleic acids and the sources for such syntheses? Typically, DNA synthesis in the malarial parasite starts at 29.5-31 hours after invasion of the red cell by the parasite. The enzyme

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responsible for chromosomal DNA replication in most eucaryotic cells is DNA polymerase α in its association with primases.⁽⁹⁾ The enzyme has recently been found and partially characterized in malaria parasites.⁽¹⁰⁾ The malarial enzyme is strongly inhibited by aphidicolin and hydroxyurea as well as mammalian DNA polymerase- α , but a monoclonal antibody recognizing an epitope on the catalytic subunit of the enzyme show on cross-reactivity with the human enzyme. These may be potentially exploitable in terms of developing novel antimalarial chemotherapy based on the structural differences between the host and parasite enzymes. The sources of nucleic acid precursors, both purine and pyrimidine bases, will be discussed later.

Purine and pyrimidine metabolism of the host red cell

The mature red cell and reticulocyte contain little or no DNA. Although reticulocytes contains RNA they cannot synthesize it from exogenous uridine or adenosine.⁽¹¹⁾ The normal red cell has a relatively limited network of purine salvage enzymes and no capacity for pyrimidine biosynthesis. The white cell contains both RNA and DNA and these can be synthesized from exogenous purine and pyrimidine bases and nucleosides. In this discussion, the metabolism of both purines and pyrimidines in the normal

red cell is mentioned only as the basis for the knowing more about perturbation that occurs following malarial infection.

The mature red cell has no capacity for *de novo* of purines and can utilize preformed purines by salvage pathways for ATP and GTP synthesis as its major requirement. The purine bases, namely adenine, hypoxanthine and guanine, can be obtained directly from serum by the normal red cells using a transport system the details of which remain to be resolved. The purine nucleosides, namely adenosine and guanosine, can be salvaged from outside the red cells by the nucleoside transport which is associated with the glucose transporter in band 4.5 of red cell membrane protein with a molecular weight of 45-60 kDa. All purines, except inosine and hypoxanthine, that enter the red cell are then metabolized to ATP and GTP by a series of enzymatic reactions which still operate in the cells. The conversion of IMP to XMP and GMP is absent in the mature red cells, although such conversion can be found in the reticulocytes by the residual activities of IMP dehydrogenase and GMP synthetase. Furthermore, there is no interconversion between ATP and GTP in the mature red cells (Fig. 1). Interestingly, the purine analogs, such as erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA), coformycin and deoxycoformycin, all potent inhibitors of red-cell adenosine deaminase, have antimalarial effect both *in vitro* against *P.falciparum* and *in vivo* against *P.knowlesi* in monkeys.⁽¹²⁻¹³⁾

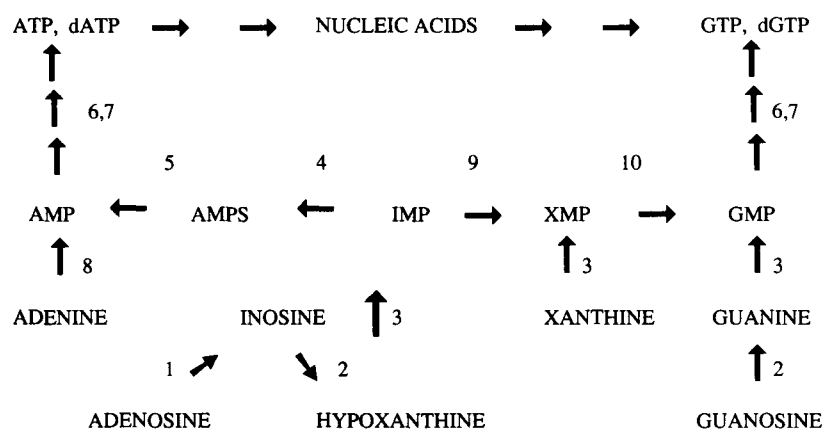


Figure 1. Purine salvage pathway in malaria parasites. The abbreviations used are as follows: AMP, adenosine monophosphate; AMPS, adenylosuccinate; ATP, adenosine triphosphate; dATP, deoxyadenosine triphosphate; GMP, guanosine monophosphate; GTP, guanosine triphosphate; dGTP, deoxyguanosine triphosphate; IMP, inosine monophosphate; XMP, xanthosine monophosphate. The numbers used indicate the enzymes as follows: 1, adenosine deaminase; 2, purine nucleoside phosphorylase; 3, hypoxanthine-xanthine-guanine phosphoribosyl transferase; 4, adenylosuccinate synthase; 5, adenylosuccinate lyase; 6, nucleoside monophosphate kinase; 7, nucleoside diphosphate kinase; 8, adenine phosphoribosyl transferase; 9, IMP dehydrogenase; 10, GMP synthase.

The red cell has no ability to *de novo* synthesis of pyrimidine because of the absence or very low levels of a number of enzymes responsible for its synthesis.⁽¹¹⁾ In addition, there is no salvage pathway for utilization of the pyrimidine nucleosides, namely uridine and thymidine, even though both nucleosides can be taken up by the cell.

Purine metabolism in the malaria parasite

We have focused on the metabolism of purines for two purposes. One is to redirect attention to the remarkable deficiency of malarial parasites with respect to the biosynthesis of purines *de novo*, and the other is to discuss the salvage pathways they employ, in particular those that have unusual properties. These novel characteristics have been realized for most parasitic protozoans.⁽¹⁴⁾

Based largely on two major technological requirements, the *in vitro* continuous culture of *P. falciparum*⁽²⁾ to permit sufficient quantities for biochemical analyses and the development of high-performance liquid chromatography (HPLC) to measure comprehensively and accurately all purine pathway intermediates,⁽¹²⁾ purine metabolism as well as pyrimidine⁽¹⁴⁾ and folate metabolism.^(15,16) Direct evidence of a lack of *de novo* purine synthesis in the intra-erythrocytic stage of the malarial parasite was provided by the lack of incorporation of radiolabelled precursor formate or glycine into purine nucleotides^(12,13) and by the absence of various enzymes of *de novo* synthesis.⁽¹⁷⁾

Malaria infection results in a much greater ability of the red cell to salvage purines, owing to the existence of enzymes responsible for the synthesis of purine nucleotides. It has been suggested that the major purine incorporated into the parasite is hypoxanthine, the most abundant purine base contained in blood.^(6,11-13) Using hypoxanthine as the radiolabelled precursor, it was found that purine nucleotide metabolism in malaria-infected red cells is different from that of host red cells in two important metabolic aspects: active synthesis of GMP, GDP and GTP from hypoxanthine via inosine 5'-monophosphate

(IMP); and the presence of an adenylosuccinate pathway to synthesize AMP, ADP and ATP from IMP.^(12,13) In addition, the parasite contains very high activity of adenosine deaminase for conversion of adenosine to inosine and hypoxanthine. It has been proposed that this enzyme may be the *in vivo* source of hypoxanthine for the malarial parasite.^(12,13) The purine salvage pathway in the malaria parasite is summarized in Fig.1. One important parasite enzyme involved in the purine salvage pathway is hypoxanthine-xanthine-guanine phosphoribosyltransferase (HGPRT) which has the ability to utilize hypoxanthine, guanine and xanthine as substrate, whereas the human host HGPRT lacks activity towards xanthine.⁽¹⁸⁾ This difference in substrate specificity leads to the development of chemotherapeutic agents specific for the parasite HGPRT (Davidson, Jr., personal communication).

Pyrimidine metabolism in the malaria parasite

As essential components of parasite development and replication, the metabolism of nucleic acid precursors is one logical component for the development of non-empirical (rational) antimalarial agents. Malaria parasites require purines and pyrimidines for DNA and RNA synthesis during exponential growth. The parasites use preformed purines, which are salvaged from the host, but they have to synthesize pyrimidine *de novo* (Fig.2). The requirement of low oxygen tension during intra-erythrocytic growth is proposed as being linked to dihydroorotate (DHO) oxidation of pyrimidine biosynthesis and a simplified electron transport system (ETS) of mitochondria containing dihydroorotate dehydrogenase (DHODase), ubiquinones, cytochromes b and c, and finally, cytochrome oxidase (Fig.3).^(19,20) The intra-erythrocytic malarial parasites derive ATP from glycolysis and do not require oxygen for energy production.⁽⁶⁾ We have focused our attention on pyrimidine biosynthetic enzymes and mitochondrial metabolism because several lines of evidence suggested that there are some key differences between the malaria parasites and the mammalian host system.

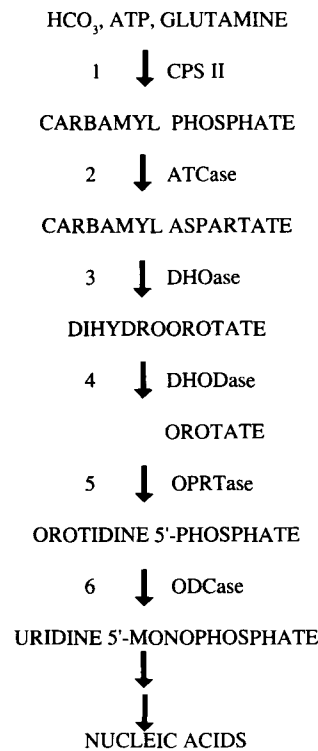


Figure 2. Pyrimidine biosynthetic pathway in malaria parasites. The abbreviations and numbers used are as follows: 1, CPS II (carbamyl phosphate synthase II) ; 2, ATCase (aspartate transcarbamylase) ; 3, DHOase (dihydroorotase) ; 4, DHODase (dihydroorotate dehydrogenase) ; 5, OPRTase (orotate phosphoribosyl transferase) ; 6, ODCase (orotidine 5'-phosphate decarboxylase).

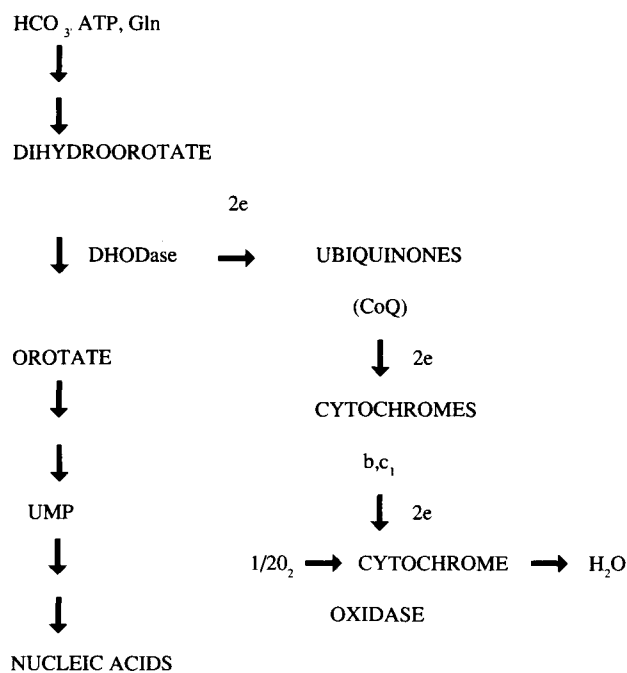


Figure 3. Proposed relationship among O₂ utilization, electron transport system and pyrimidine biosynthesis.

Evidence of functional pyrimidine biosynthesis *de novo* in the intra-erythrocytic stage of the malaria parasite comes largely from these lines of investigation. Firstly, *P.falciparum* growing *in vitro* culture can incorporate radioactive bicarbonate into pyrimidine bases of DNA and RNA. In the parasite, it has been demonstrated that there is very little incorporation of orotate, uridine and thymidine into DNA and RNA, whereas these pyrimidines can be easily taken up by the *P.falciparum*-infected red cell (Krungkrai, unpublished results).⁽¹¹⁾ Secondly, the enzymes responsible for salvaging preformed pyrimidine bases have never been demonstrated; those enzymes are thymidine kinase,⁽¹⁴⁾ uridine kinase and cytosine kinase.⁽²¹⁾ Thirdly, all the enzymes required for uridine 5'-monophosphate (UMP) synthesis *de novo* (see Fig.2) have been detected in extracts from several species of *Plasmodium*.⁽²⁰⁻²⁵⁾

The first three enzyme of pyrimidine biosynthesis [carbamyl phosphate synthase II (CPS II), aspartate transcarbamylase (ATCase), dihydroorotase (DHOase)]

in the malaria parasites *P.berghei* and *P.falciparum* are found to be separated on individual proteins by using a Superose 12 gel filtration column on a fast protein liquid chromatography (FPLC) system in the presence of stabilizing agents and mixtures of protease inhibitors. In a control experiment with host mammalian enzymes under the same conditions, the three enzymatic activities were eluted at the same position on the FPLC system of apparent $M_r > 670$ kDa.⁽²²⁾ Our result is similar to what is found in most prokaryotic systems but differs from the analogous mammalian systems in which the first three enzymes are associated with a single multifunctional protein.⁽²⁶⁾ We have extensively characterized the third enzyme DHOase catalyzing the reversible reaction of carbamyl aspartate (CA) and dihydroorotate (DHO), and the mechanism of catalytic action has been proposed as follows: the action of an active-site Zn^{2+} ion which can coordinate to a putative tetrahedral intermediate form following attack by the ureido amino function at the β -carboxyl group of CA (in the forward direction) or following attack by water at this carboxyl group (in the backward direction) (Fig.4.)⁽²²⁾

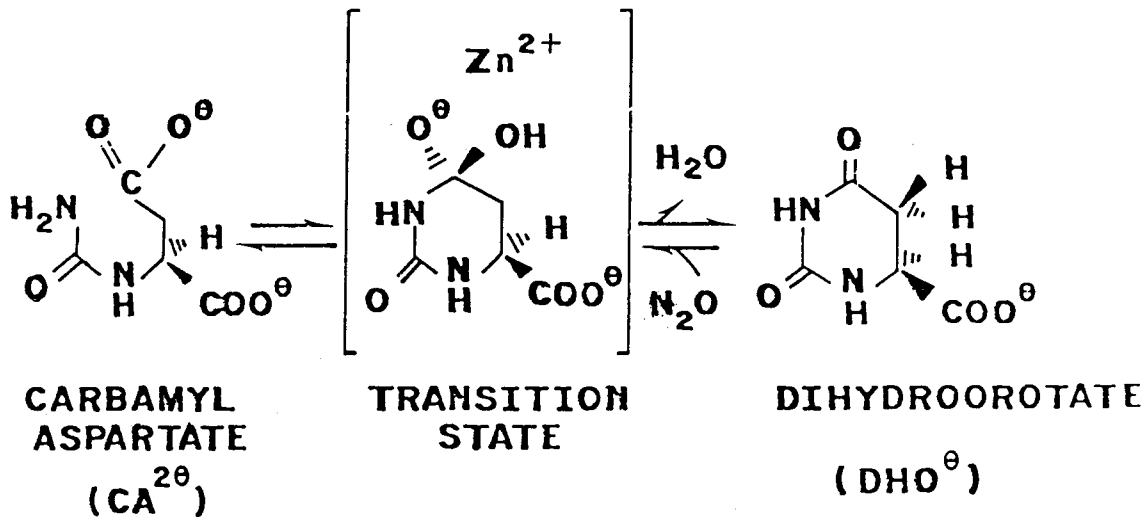


Figure 4. Proposed malarial DHOase enzymatic catalysis.

The fourth enzyme of the pathway, dihydroorotate dehydrogenase (DHODase), has been shown to be a particulate protein and is extensively characterized.⁽²⁰⁾ By analogy to other systems the DHODase is expected to be associated with the outer surface of the inner membrane of the mitochondria in the malaria parasite. The last two enzymes, orotate phosphoribosyltransferase (OPRTase) and orotidine 5'-phosphate decarboxylase (ODCase), catalyze the final steps of *de novo* synthesis of uridine 5'-monophosphate (UMP) in mammalian cells and exist as bifunctional protein,⁽²⁶⁾ but in malaria parasites these enzymes have been described as discrete entities.⁽²³⁾ From the lines of evidence, it appears that *de novo* biosynthesis of pyrimidine in malaria parasites is catalyzed by six discrete enzyme activities, different from that in the analogous mammalian process. Furthermore, while the malaria parasites are totally dependent on the *de novo* synthesis because they lack relevant salvage enzymes (for instance, thymidine kinase), in contrast, the host mammalian cells can either use preformed pyrimidine bases and nucleosides or synthesize their own. These key differences suggest that the malaria parasites may be sensitive to novel antimalarial drugs that act on pyrimidine *de novo* synthesis. One such compound, hydroxynaphthoquinone A566C, possibly inhibiting DHODase activity, is being developed and tested in a human trial.⁽²⁷⁾

Pyrimidine biosynthetic enzymes as drug targets

In order to exploit fully these pharmacological targets, it is necessary to pursue detailed mechanistic and structural characterization of these enzymes of pyrimidine biosynthesis. We have studied novel forms of DHOase and DHODase from malarial parasites. The malarial DHOase has a monomeric structure with a native molecular mass of 38 kDa. Notwithstanding the marked physical differences, the characterization of kinetic parameters of the malarial DHOase suggest that the protozoan, mammalian and bacterial DHOase enzymes share a common catalytic mechanism.⁽²²⁾

The malarial DHODase has been purified to apparent homogeneity by detergent solubilization followed by anion-exchange, dye-agarose affinity and gel filtration chromatography. It is membrane-bound and active in monomeric form with a molecular weight of 55 kDa. The DHODase contains about 2 mol of iron and 2 mol of acid-labile sulfur per mole of enzyme, and does not contain a flavin cofactor. It is interesting to note that the optimum activity of DHODase requires ubiquinone coenzymes CoQ₆, CoQ₇, CoQ₉ or CoQ₁₀. The cofactor and kinetic analyses of the enzyme-catalyzed reaction suggest that the enzyme catalyzes DHO oxidation through the inter-

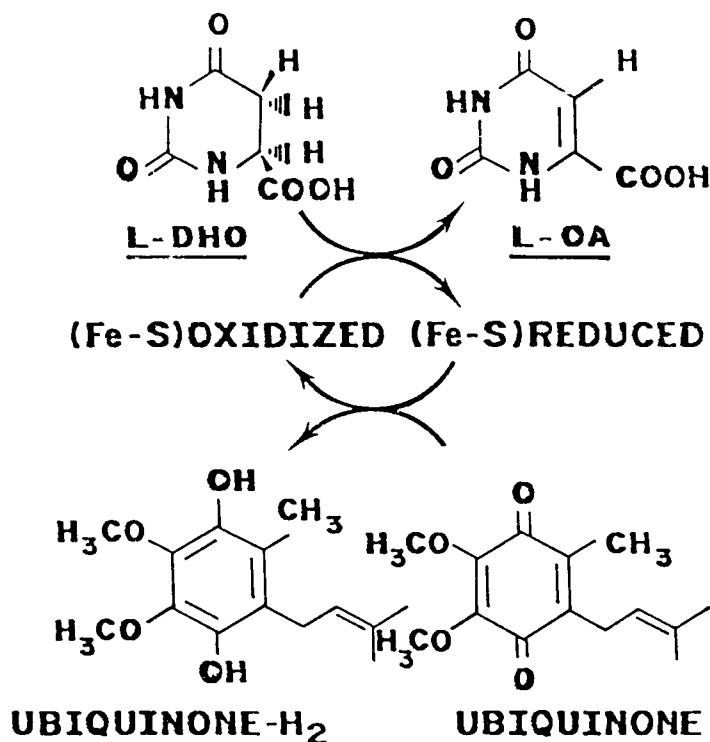


Figure 5. Proposed malarial DHODase enzymatic catalysis.

mediary of a single [2Fe-2S] iron-sulfur cluster with ubiquinone acting as an obligatory electron transfer (Fig.5).⁽²⁰⁾ Our results support the hypothesis of the linkage of pyrimidine biosynthesis to the electron transport system and limited oxygen utilization in malaria parasites by DHODase via ubiquinones.

Recently, we have reported that there is a cytosolic form of DHODase which catalyzes the reduction of orotate to DHO. The physiological significance of this form remains to be elucidated.⁽²⁸⁾ Interestingly, the purified malarial DHODase has ability to generate a superoxide radical during oxidation of dihydroorotate to orotate as is evidenced by the following. The rate of dichlorophenol-indolphenol, cytochrome c and nitroblue tetrazolium reductions by DHODase were significantly inhibited by enzyme superoxide dismutase (SOD) or theonyltrifluoroacetone (specific iron chelator of the enzyme). This was also confirmed by activity staining on non-denaturing polyacrylamide gel electrophoresis of the purified enzyme. Our results are consistent with the finding of the existence of manganese-SOD in the mitochondria of the parasite, suggesting that the production of the superoxide radical may occur *in vivo*.⁽²⁹⁾

There is a need for new antimalarials with modes of action different from those of drugs currently in use or even promising drugs since the spread of multi drug-resistant *P.falciparum* is very rapid. Recent work has modified our knowledge about the malarial enzymes of pyrimidine biosynthesis, these enzymes may be good targets for the development of antimalarial drugs. We have carried out a systematic study testing known compounds affecting the enzymes of the pathway and also having potent antimalarial activity against *P.falciparum* grown *in vitro* and *P.berghei* in mice. Orotate and a series of five substituted derivatives are found to inhibit competitively the purified DHOase and DHODase enzymes in the malarial parasite (Table 2). As mentioned previously, the malarial DHOase catalyzes the reversible reactions of CA and DHO. The following results on the kinetic parameters and inhibitory effect of orotate analogs on the malarial DHOase are from the reaction of DHO to CA (the backward reaction). The order of effectiveness as inhibitors for malarial DHOase is as follows: F-OA>HN₂-OA, CH₃-OA>OA>Br-OA>I-OA, whereas OA, F-OA and NH₂-OA are the most effective inhibitors for malarial DHODase.^(20,22,30)

Table 2. Effect of orotate analogs on the malarial DHOase and DHODase enzymes.

Compounds	Symbols	DHOase		DHODase	
		K _m (μM)	K _i (μM)	K _m (μM)	K _i (μM)
Substrates					
carbaryl aspartate	CA	-	-	-	-
dihydroorotate	DHO	18.5	-	8.0	-
Inhibitors					
orotate	OA	-	860	-	31
5-fluoorotate	F-OA	-	65	-	35
5-aminoorotate	NH ₂ -OA	-	142	-	44
5-methylorotate	CH ₃ -OA	-	166	-	158
5-bromoorotate	Br-OA	-	2200	-	380
5-iodoorotate	I-OA	-	>3500	-	464

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As may be observed from Table 3, F-OA has the most potent antimalarial activity against both *P. falciparum* *in vitro* and *P. berghei* in mice. CH₃-OA and NH₂-OA also have potent antimalarial activity. Our results on the

inhibitory effect and antimalarial activity of orotate analogs suggest that the two enzymes, DHOase and DHODase, in the malaria parasite may be good targets for the design of new classes of antimalarial drugs.⁽³⁰⁻³²⁾

Table 3. Antimalarial activity of orotate analogs against *P. falciparum* grown *in vitro* and *P. berghei* in mice.

Orotate analogs	<i>P. falciparum</i> [IC ₉₀ (μM)]	<i>P. berghei</i> [ED ₉₀ (mg/kg)]
OA	N.E.	N.E.
F-OA	0.06	2.5
CH ₃ -OA	6.0	>25.0
NH ₂ -OA	50.0	<10.0
Br-OA	N.E.	not test
I-OA	N.E.	not test

IC₉₀ : concentration of compound producing 90% inhibition of *P. falciparum* grown *in vitro*.

ED₉₀ : dose of compound giving 90% inhibition of parasitemia for 4-day suppressive test in mice infected with *P. berghei*

N.E. : no effect on parasite morphology at 1.0 mM for *P. falciparum* or at dose of 25 mg/kg for *P. berghei*.

In collaboration with Drs. A. Cerami and G.B. Henderson (Rockefeller University, New York), we have synthesized a quinone analog 2-chloromethyl-3-methyl-1,4-naphthoquinone, a mechanism-based inhibitor for malarial DHODase. Some kinetic parameters of the inhibitory effect against the purified DHODase have been identified. The quinone has K_i and K_{inh} values of 7 μM and 0.86 min⁻¹, respectively. It shows moderate antimalarial activity against *P. falciparum* *in vitro* with 50% inhibitory concentration of 10 μM. *In vivo* antimalarial activity of the quinone against *P. berghei* infected mice at a dose of 17.5 mg/kg body weight daily for four days cleared parasitemia in infected mice, in comparison with chloroquine therapy with a standard dose of 25 mg/kg.^(31,32) The efficacy of quinone is somewhat comparable to that of chloroquine. Series of the quinone analogs as mechanism-based inhibitors are being studied.

Conclusion

Much effort is being spent to develop malaria vaccines, but despite the vast amount of knowledge gained about the parasite's life-cycle and many surface antigens, the prospects of success are as yet uncertain. The vaccine

candidates have not been as immunogenic as was hoped. Our results, together with that of other investigators, suggest that the pyrimidine biosynthetic pathway and mitochondrial metabolism may be a promising target for the design of antimalarial drugs. The attractiveness of these targets will become clearer as the identities and specificities of all enzymes in the pathway become clearer.

A perspective

At present, the utility of most antimalarial drugs is threatened or impaired by the emergence of multidrug-resistant malaria parasites. Therefore, improvements in chemotherapy for treatment of malaria are urgently required. The new developments will require better understanding of the mechanisms of drug action and drug resistance and also the basic biochemistry of the malaria parasite.

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References

1. Tropical Disease Research, TDR Tenth Programme Report; 1989-1990, UNDP/World Bank/WHO special Programme for Research and Training in Tropical Diseases. World Health Organization, Geneva, 1990.
2. Trager W, Jensen JB. Human malaria parasites in continuous culture. *Science* 1976 Aug;193(4254): 673-5
3. Krungkrai J, Yuthavong Y. Enhanced Ca^{2+} uptake by mouse erythrocytes in malarial *Plasmodium Berghei* infection. *Mol Biochem Parasitol* 1983 Mar;7(3): 227-35
4. Pouvelle B, Spiegel R, Hsiao L, Howard RJ, Morris RL, Thomas AP, Taraschi TF. Direct access to serum macromolecules by intraerythrocytic malaria parasites. *Nature* 1991 Sep 5; 353(6339): 73-5
5. Krungkrai J. Biochemistry of malaria I : folate and cobalamin and mechanism of pyrimethamine resistance in *Plasmodium Falciparum*. *Chula Med J* 1991 Feb; 35(2): 105-11
6. Sherman IW. Biochemistry of *Plasmodium* (malarial parasites). *Microbiol Rev* 1979 Dec; 43(4): 453-95
7. Hyde JE, Goman M, Hall R, Osland A, Hope IA, Langsley G, Zolg JW, Scaife JG. Characterisation and translation studies of messenger RNA from the human malaria parasite *Plasmodium Falciparum* and construction of a cDNA library. *Mol Biochem Parasitol* 1984 Mar; 10(3): 269-85
8. Janse CJ. Chromosome size polymorphism and DNA rearrangements in *Plasmodium*. *Parasitology Today* 1993 Jan; 9(2) : 19-22
9. Lehman IR, Kaguni LS. DNA polymerase . *J Biol Chem* 1989 Mar; 264(8) : 4265-8
10. De Vries E, Stam JG, Franssen FF, van der Vliet. PC, Overeulve JP. Purification and characterization of DNA polymerases from *Plasmodium Berghei*. *Mol Biochem Parasitol* 1991 Apr; 45(2) : 223-32
11. Gero AM, O'Sullivan WJ. Purines and pyrimidines in malarial parasites. *Blood Cells* 1990; 16(2-3) : 467-84
12. Webster HK, Whaun JM. Purine metabolism during continuous erythrocytic culture of human malaria parasite (*P. Falciparum*). In : *The Red Cell : Fifth Ann Arbor Conference*. New York : Alan R. Liss, 1981: 557-73
13. Daddona PE, Wiesmann WP, Lambros C, Kelley WN, Webster HK. Human malaria parasite adenosine deaminase. Characterization in host enzyme-deficient erythrocyte culture. *J Biol Chem* 1984 Feb; 259-(3) : 1472-5
14. Krungkrai J, Yuthavong Y, Webster HK. High-performance liquid chromatographic assay for thymidylate synthase from the human parasite. *J Chromatogr* 1989; 487(1) : 51-9
15. Krungkrai J, Webster KH, Yuthavong Y. De Novo and salvage biosynthesis of pteroylpentaglutamate in the human malaria parasite, *Plasmodium Falciparum*. *Mol Biochem Parasitol* 1989; 32(1) : 25-38
16. Krungkrai J, Webster KH, Yuthavong Y. Folate and cobalamin metabolism in *Plasmodium Falciparum*. *Parasitology Today* 1990 Dec; 6(12) : 388-91
17. Hammond DJ, Gutteridge WE. Purine and pyrimidine metabolism in the Trypanosomatidae. *Mol Biochem Parasitol* 1984 Nov; 13(3) : 243-61
18. Queen SA, Vander Jagt D, Reyes P. Properties and substrate specificity of a purine phosphoribosyltransferase from the human malarial parasite, *Plasmodium Falciparum*. *Mol Biochem Parasitol* 1988; 30 : 123-34
19. Krungkrai J, Krungkrai SR, Bhumiratana A. *Plasmodium Berghei* : Purification and characterization of mitochondrial cytochrome c oxidase. *Exp Parasitol* 1993. (in press.)
20. Krungkrai J, Cerami A, Henderson GB. Purification and characterization of digydroorotate dehydrogenase from the rodent malarial parasite *Plasmodium berghei*. *Biochemistry* 1991 Feb; 30(7) : 1934-9
21. Hill B, Kilsby J, Rogerson GW, McIntosh RT, Ginger CD. The enzymes of pyrimidine biosynthesis in a range of parasitic protozoa and helminths. *Mol Biochem Parasitol* 1981 Feb; 2(3-4) : 123-34
22. Krungkrai J, Cerami A, Henderson GB. Pyrimidine biosynthesis in parasitic protozoan : purification of a monofunctional dihydroorotase from *Plasmodium Berghei* and *Crithidia Fasciculata*. *Biochemistry* 1990 Jul; 29(26) : 6270-5
23. Rathod PK, Reyes P. Orotidylate-metabolizing enzymes of the human malarial parasite, *Plasmodium Falciparum*, differ from host cell enzymes. *J Biol Chem* 1983 Mar; 258(5) 2852-5
24. Gero AM, Tetley K, Coombs GH, Phillips RS. Dihydroorotate dehydrogenase, orotate phosphoribosyltransferase and orotidine 5'-phosphate decarboxylase in *Plasmodium Falciparum*. *Trans Roy Soc Trop Med Hyg* 1981; 75 (5) : 719-20
25. Gero AM, Brown GV, O'sullivan WJ. Pyrimidine de novo synthesis during the life cycle of the

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- intraerythrocytic stage of *Plasmodium Falciparum*. *J Parasitol* 1984 Aug; 70(4): 536-41
26. Jones ME. Pyrimidine nucleotide biosynthesis in animals : genes, enzymes, and regulation of UMP biosynthesis. *Annu Rev Biochem* 1980; 49: 253-79
27. Gutteridge WE. Parasite vaccines versus anti-parasitic drugs : rivals or running mates? *Parasitology* 1989; 98 : S87-S97
28. Krungkrai J. A novel form of orotate reductase that converts orotate to dihydroorotate in *Plasmodium Falciparum* and *Plasmodium Berghei*. *Comp Biochem Physiol* 1993. (in press.)
29. Krungkrai J. Malarial dihydroorotate dehydrogenase mediates superoxide radical production. *Biochem Int* 1991 Jul; 24(5) : 833-9
30. Krungkrai J, Krungkrai SR, Phakanont K. Antimalarial activity of orotate analogs that inhibit dihydroorotase and dihydroorotate dehydrogenase. *Biochem Pharmacol* 1992 Mar; 43(6) : 1295-301
31. Krungkrai J, Krungkrai SR, Suriyamonthon N, Phakanont K, Bhumiratana A. Biochemical basis of pyrimidine biosynthesis and chemotherapy in human malaria parasite. *Proceedings of the Eighth Japan-China Joint Seminar on Parasitic Diseases, Tokyo, 1992* : 13-18
32. Krungkrai J. Dihydroorotase and dihydroorotate dehydrogenase as a target for antimalarial drugs. *Drugs of the Future* 1993. (in press.)

หมายเหตุ

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