CHAPTER I
INTRODRUCTION

While we are worrying about new infectious diseases like AIDS, malaria remains to be a major public health problem, especially for a tropical and subtropical countries like South-East Asia and Africa. Malaria is already estimated to have killed between 1.5 and 2.7 million people every year.[1] Up to 1 million of deaths from malaria are among children younger than five years old.[2] Furthermore it is estimated that about 40% of the world's population is at risk from this disease according to the World Health Organization (WHO).[3]

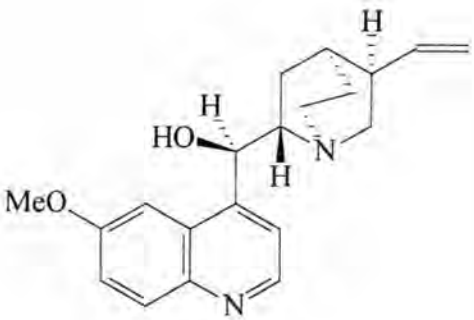
The cause of malaria in human is one of the four species of protozoan parasites of the *Plasmodium* genus.[4-6] There are *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale* and *Plasmodium malariae.* Each species presents slightly different clinical symptoms. *Plasmodium falciparum* is the most pernicious, since it causes the majority of malaria-related morbidity and mortality, and geographically the most widespread of the four. While other *Plasmodium* species specifically infect a variety of birds, reptiles, amphibians, and mammals.

Parasites are transmitted from one person to another by the female anopheles mosquito. These mosquitoes are present in almost all countries in the tropics and subtropics and they bite during nighttime hours. When an infected mosquito bites a person, parasites are passed into the blood system of the human victim and then travel to liver tissue where they invade the cells and multiply. After 9-15 days they return to the blood and penetrate the red blood cells, where they again multiply and begin destroying the red blood cells.

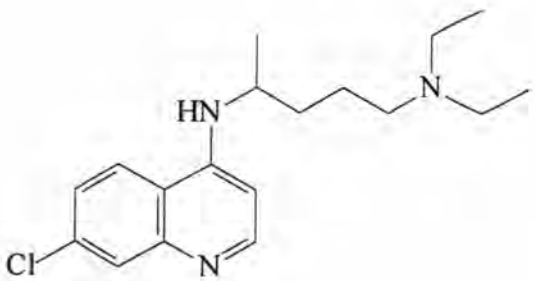
The signs and symptoms of malaria sickness may occur after the patients were infected for 10-15 days. Early symptoms include fever and other symptoms as headache, back pain, chills, muscle ache, nausea and sometime vomiting, diarrahea, and cough. Of all the four species of protozoan parasites, the only *Plasmodium falciparum* malaria can progress rapidly to the cerebral stage, where the infected red cells obstruct the blood vessels in the brain. Untreated cases can give rise to coma, renal failure, liver failure, pulmonary edema, convulsions, and eventually death. Even though infections with *Plasmodium vivax* and *Plasmodium ovale* often cause less

serious ailment. The parasites may remain dormant in the liver for many months causing a reappearance of symptoms months or even years later.

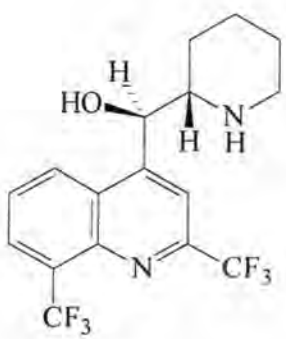
Malaria have been treated by anti-malarial drugs, for example, quinine, chloroquine, mefloquine and artemisinin[6] (Scheme 1.1). Quinine, for three hundred years, was the only known effective treatment for this life-threatening infectious disease. Quinine was isolated from the stem bark of the cinchona tree, *Cinchona officinalis* and other *Cinchona* species, a native plant from South America and was the first drug to be used against malaria. Quinine is still produced as the flavouring agent for tonic water[7] although it is now of little use against malaria. Chloroquine and mefloqine are synthetic analogs of quinine which are not much used nowadays due to the development of drug resistance. The weed *Artemisia annua* (sweet wormwood, sweet annie) has been used for many centuries in Chinese herbal medicine as a treatment for fever and malaria.[8-9] In 1971, Chinese chemists isolated artemisinin from the leafy portions of the plant.[8,10] Artemisinin showed good anti-malarial activity and it is in fact one of the most effective drugs currently used.



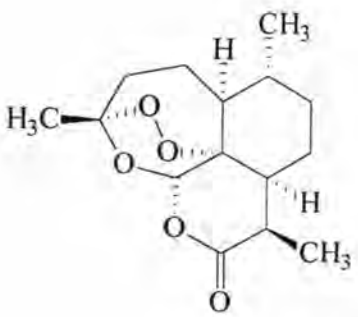
Quinine



Chloroquine



Mefloquine

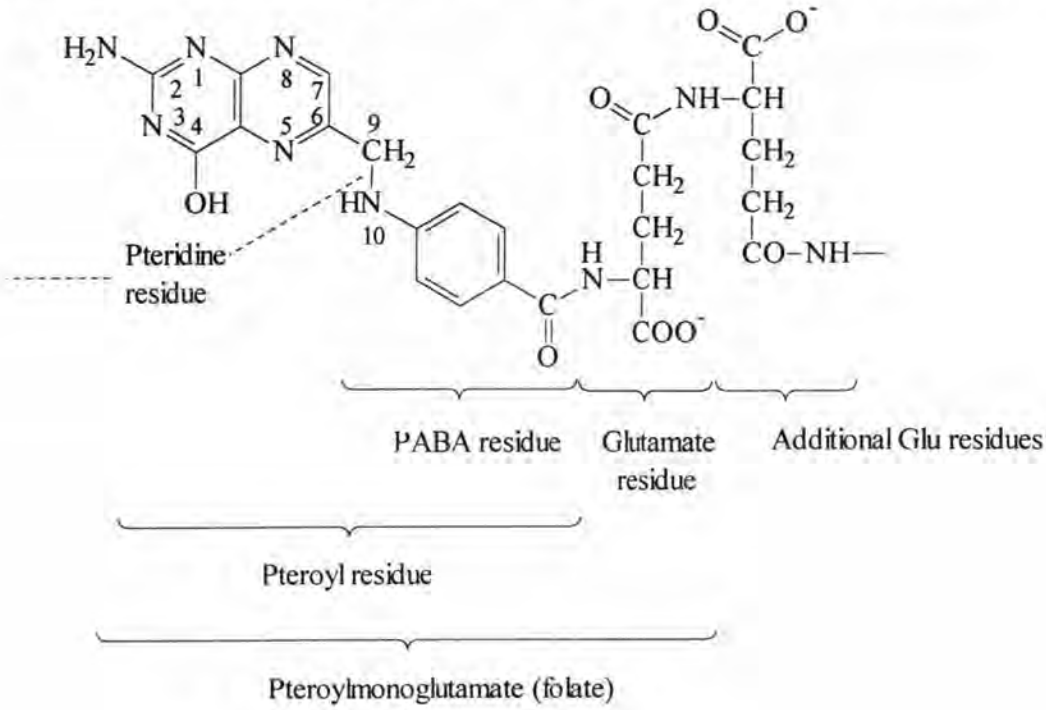


Artemisnin

Scheme 1.1 Anti-malarial drugs

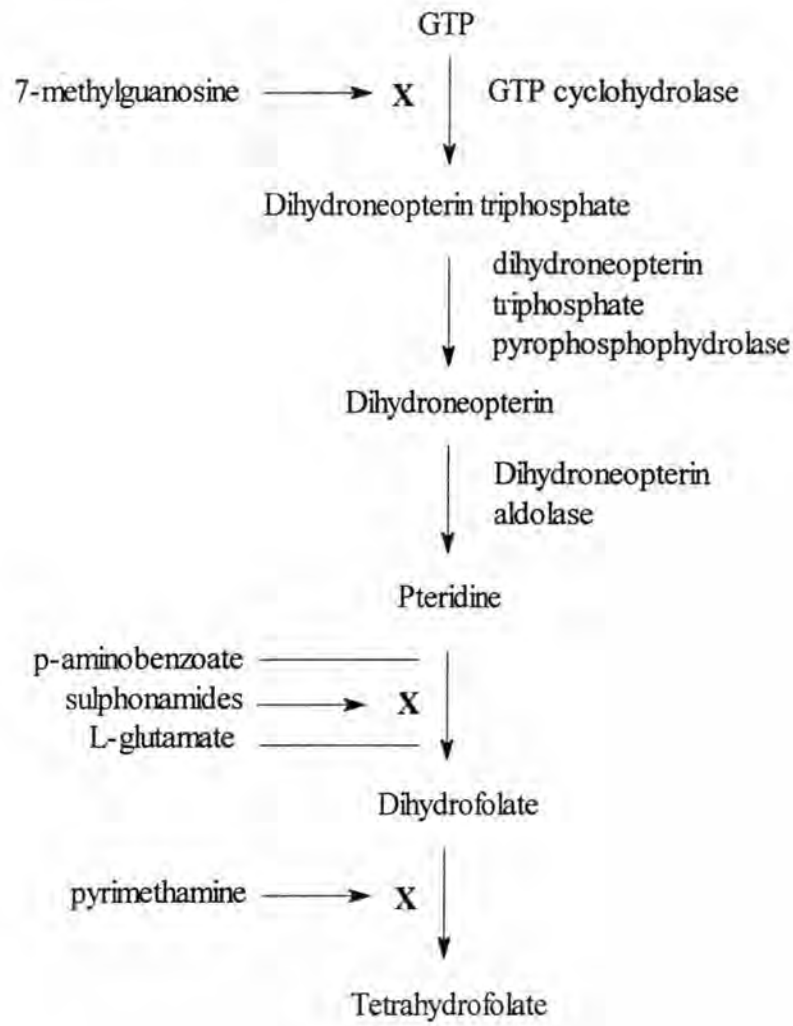
However, we have one question that why malaria still exists. Many studies suggested that a major cause for persistency of malaria is that the malarial parasites can develop resistance to many anti-malarial drugs that were used for a long time, particularly chloroquine and quinine. In addition, many metabolic pathways of malaria were similar to human, therefore many compounds which are toxic to the parasites could not be developed to drugs because they are also toxic to human. The lack of knowledge about malaria was another cause because most of scientists paid more interests on bacteria or virus more than to protozoa.

From the study of the life cycle of malarial parasites, it was found that many metabolic pathway of the parasite may be selectively inhibited, and thus are potential targets for development of antimalarial drugs. One of the most thoroughly investigated is the folate pathway. It is folate or pteroylglutamic acid that is the necessary compound to the living of malarial parasites, human and animals.[11] Folate is normally found in vegetables and green plants. Folate joins two cycles sharing a common step. One cycle is the methionine synthesis and the other is the thymidylate synthesis cycles, which produce methionine, a component of protein, and thymidylate, a building block for DNA synthesis respectively. Folate consists of three parts, which are heterobicylic pteridine ring, *p*-aminobenzoic acid and glutamic acid (Scheme 1.2).



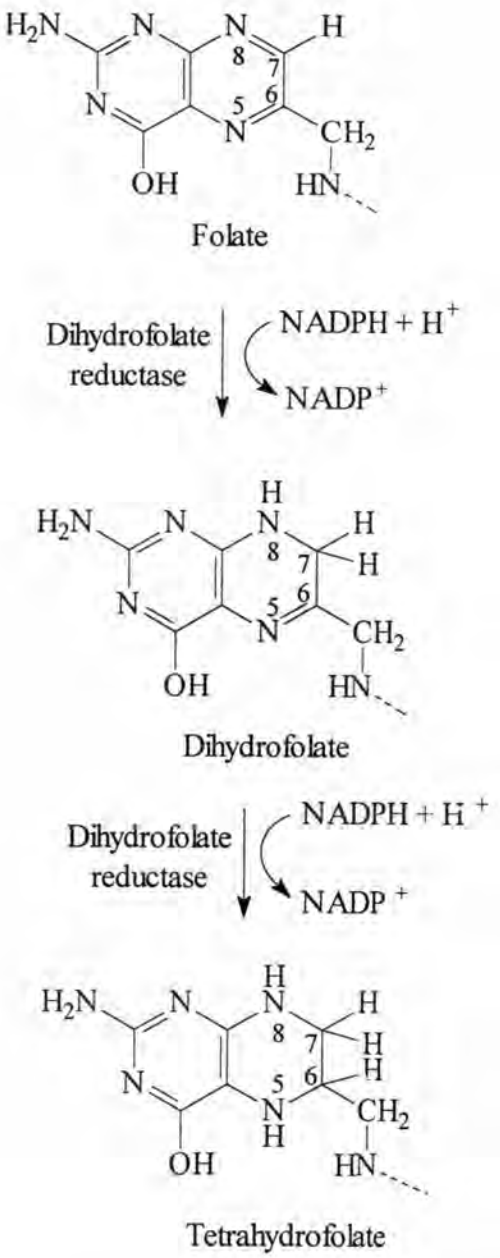
Scheme 1.2 Structure of folate

While human can not synthesize folate by themselves, the malaria parasites can do it by using enzymes. Moreover, malaria can take and use folate from external sources. Synthesis of tetrahydrofolate in falciparum malaria starts from guanosine triphosphate (GTP) which is converted to dihydroneopterin, an intermediate of folate synthesis by GTP cyclohydrolase[12] (Scheme 1.3).



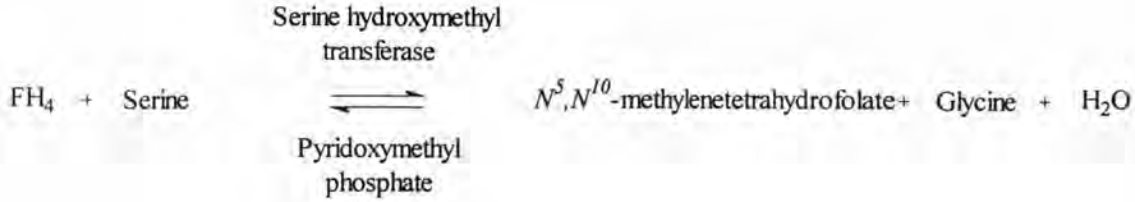
Scheme 1.3 Folate pathway in malaria disease

Dihydrofolate reductase (DHFR) is a key enzyme in the folate pathway which is essential for conversion of folate to tetrahydrofolate (FH_4)[13] by a two-step reduction (Scheme 1.4). Tetrahydrofolate is an essential precursor of thymidylate synthesis and thus is essential to the living of malaria.



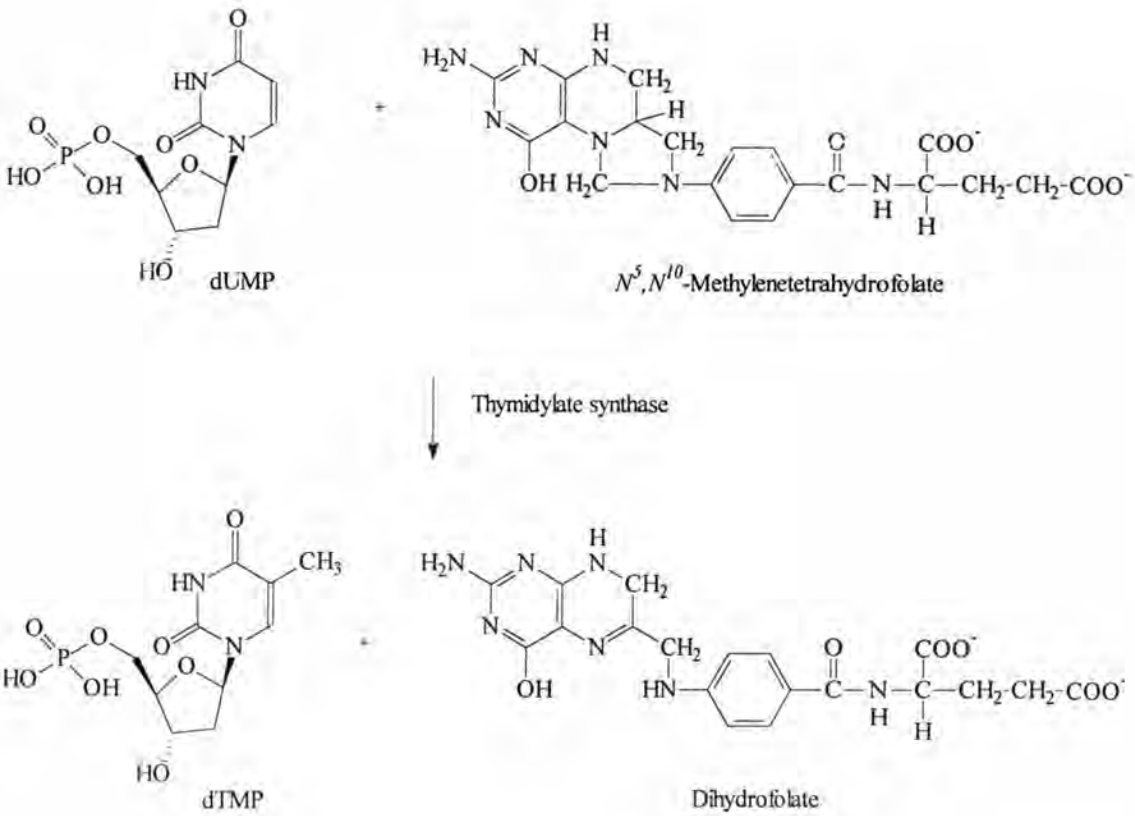
Scheme 1.4 Reduction of folate to tetrahydrofolate

In the synthesis of thymidylate, tetrahydrofolate is reacted with serine to generate N^5,N^{10}-methylenetetrahydrofolate,[14] a one-carbon transfer reagent as shown in Scheme 1.5.



Scheme 1.5 Reaction of tetrahydrofolate and serine

N^5,N^{10}-Methylenetetrahydrofolate is then used in thymidylate synthesis as shown in Scheme 1.6.

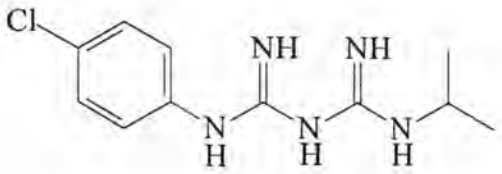


Scheme 1.6 Thymidylate synthesis

If we could stop any stages of the synthesis of FH_4 cycle, it will not be produced and the thymidylate synthesis process will be inhibited altogether.

Some antimalarial drugs in the market, acted by inhibiting the dihydrofolate reductase domain of *Plamodium falciparum* bifunctional dihydrofolate reductase-thymidylate synthase enzyme (DHFR-TS),[15] which is one of the few well-defined targets in malarial chemotherapy. These drugs were called collectively as antifolates.

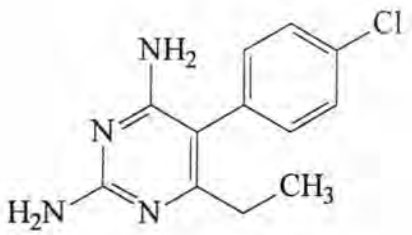
Proguanil,[6] for example, was an anti-malarial drug and had been one of the most successful chemotherapeutic agents for treatment malaria disease in human (Scheme 1.7).



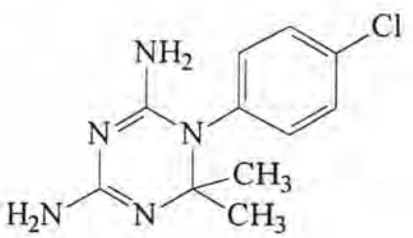
Proguanil

Scheme 1.7 Structure of proguanil

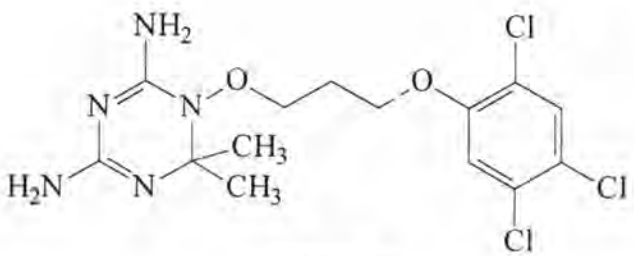
After entering the body, proguanil is metabolized to cycloguanil, the actual antifolate.[16] There are also other anti-malarial drugs worked by inhibition of the dihydrofolate reductase enzyme. Examples of such inhibitors include 2,4-diaminopydrimidines such as pyrimethamine[11] (Pyr) and 2,4-diamino-1,2-dihydro-1,3,5-triazines such as cycloguanil[11] (Cyc) and WR99210[17] (Scheme 1.8)



Pyrimethamine (Pyr)



Cycloguanil (Cyc)



WR99210

Scheme 1.8 Samples of antifolate

Unfortunately these drugs are virtually useless now due to the development of resistance of the parasites. So it is necessary to discover and develop new anti-malarial aimed at combating the emerging resistant parasites.

It was shown that *Plasmodium falciparum* could develop resistance to antifolates by point mutation of some amino acid residues in the DHFR enzyme.[18-27] For example, the mutation of one or more amino acid residues at position 16, 51, 59, 108, and 164 of pfDHFR were identified to be involved in antifolate resistance. The studies found that mutant pfDHFRs involving mutations at amino acid residues 51, 59, 108, and 164 conferred cross-resistance to both Pyr and Cyc since the structures of them were very closely similar. While those involving mutations at residue 16 (A16V) including the A16V+S108T double mutation were resistant to Cyc but susceptible to Pyr. Furthermore WR99210, 4,6-diamino-1,2-dihydro-1,3,5-triazine whose structure was closely related to Cyc, was highly effective against the A16V+S108T mutant pfDHFR. This information was supported by the inhibition constant (K_i) values (Table 1.1).

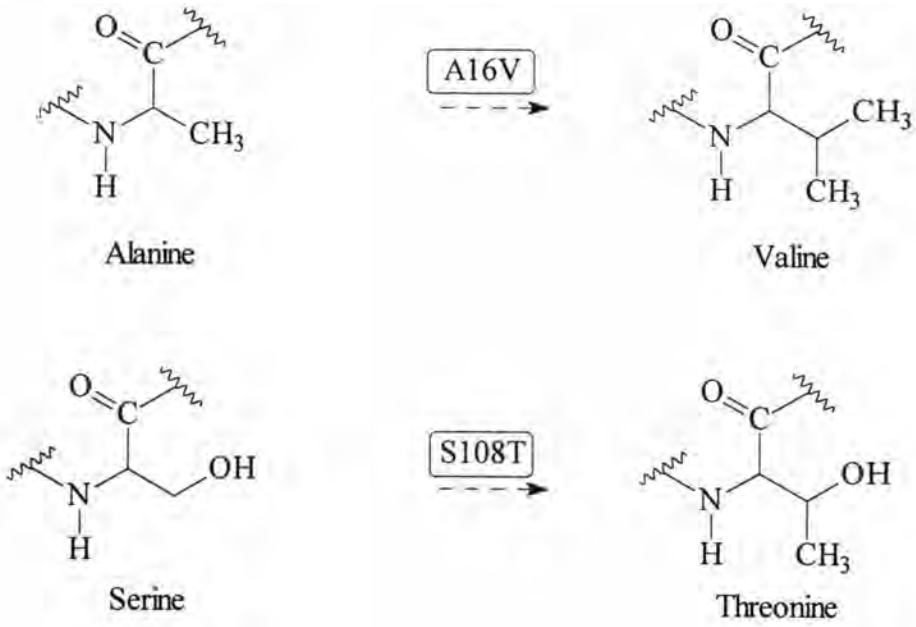
Table 1.1 Inhibition Constants (K_i) of Pyr, Cyc and WR99210 against the Wild-Type and A16V+S108T DHFRs of *P. flaciparum.*

Compound	K_i (wt)[a] (nM)	K_i (mut.)[b] (nM)	K_i (mut.)/ K_i (wt)
Pyr	1.5 ± 0.2[c]	3.6 ± 0.3[c]	2.4
Cyc	1.5 ± 0.3[c]	1,314.0 ± 16[c]	876
WR99210	0.5 ± 0.1[d]	2.4 ± 0.4[d]	4.8

[a]Wild-type. [b]A16V+S108T mutant pfDHFR. [c]Data from ref 28. [d]Data from ref 29.

Data in Table 1.1 indicated that the inhibition constant (K_i) values of Pyr, Cyc and WR99210 to wild-type enzyme were almost indifferent. With A16V+S108T pfDHFR mutant enzyme with mutation of amino acid at position 16 from alanine to valine and at position 108 from serine to threonine (Scheme 1.9), the binding with Cyc was decreased as shown by a K_i value of 1314 ± 16 nM as compared to the wild-type enzyme (K_i = 1.5 ± 0.3 nM). Interestingly the binding affinities of Pyr and

WR99210 with A16V+S108T mutant enzyme were only slightly decreased from the wild-type.



Scheme 1.9 Changing of amino acids in A16V+S108T mutant pfDHFR

Recently, a three-dimensional homology model of pfDHFR was constructed to help the understanding of the structural basis of antifolate resistance in malaria.[29] The model structures of the complexes between Pyr, Cyc and WR99210 with the wild-type, A61V, S108T, and A16V+S108T mutant enzymes were studied by Rastellli *et al*[29] as shown in Figure 1.1.

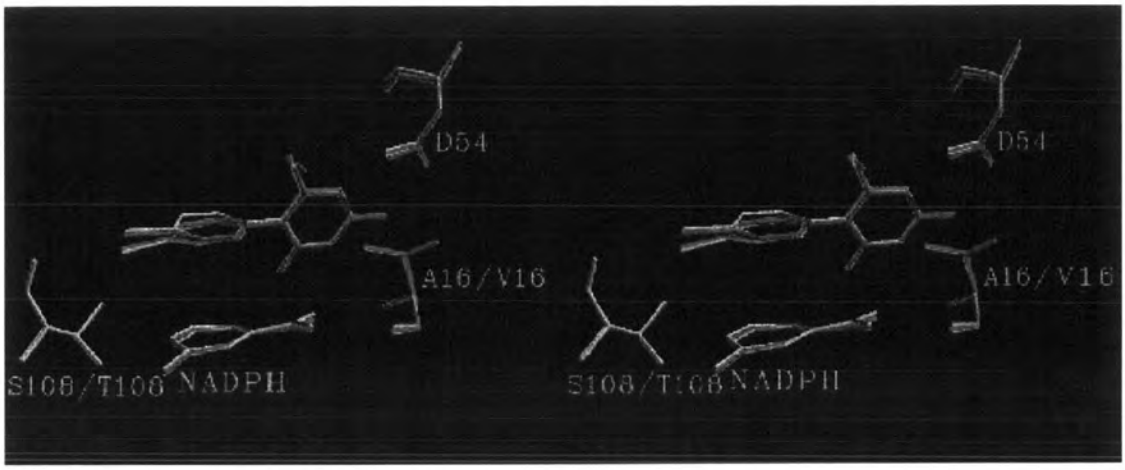
Figure 1.1A illustrated the molecular dynamics simulation of the Pyr complex with A16V mutant pfDHFR, which showed mininal changes of orientation and binding interactions with relation to that of the wild-type enzyme complex. On the contrary, the complex between Cyc and the A16V mutant pfDHFR was significantly different from the complex with the wild-type enzyme. The Cyc was displaced from its position in the wild-type complex because of the steric conflict with valine in A16V mutant (Figure 1.1B). Superimposition between Cyc and Pyr in the wild-type complexes revealed that one of the two 2,2-methyl substituents of Cyc was located much closer to the methyl of the A16 side chain with relation to the 6-ethyl substituent of Pyr. Consequently, the steric demand imposed by the A16V mutation should be much more pronounced for Cyc than for Pyr, although hydrogen-bonding

interactions with D54, I14, and I164 were still present. Intuitively WR99210 was predicted to bind the A16V mutant in the same orientation with Cyc since the 4,6-diamino-1,2-dihydro-1,3,5-triazine rings of WR99210 and Cyc were identical. As a result, the 2,2-dimethyl substituents of WR99210 were expected to be in steric conflict with the valine side chain of the A16V mutant pfDHFR too.

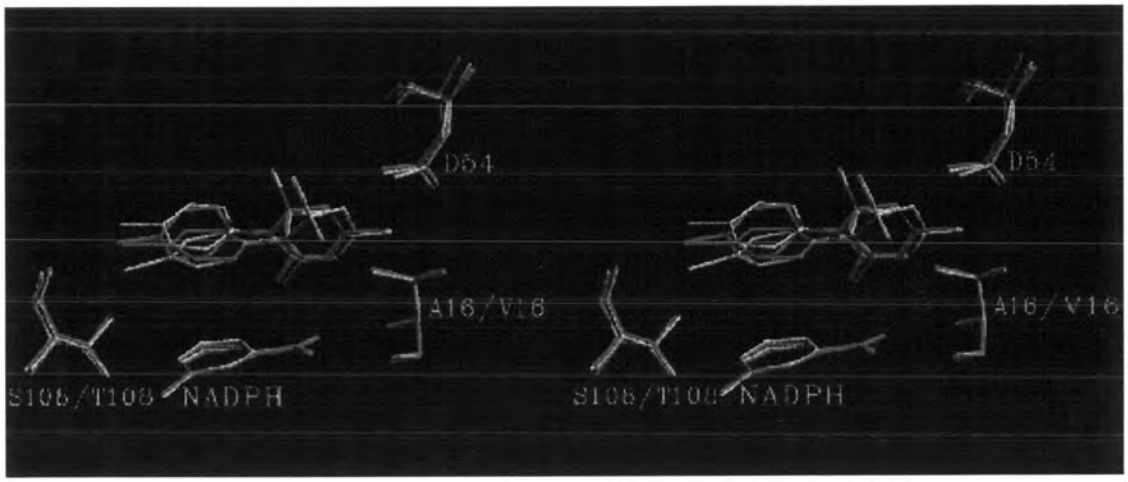
However, the model (Figure 1.1C) indicated that the flexible trichlorophenoxypropyloxy substituent of WR99210 was not forced to change its orientation significantly in the A16V mutant when compared to the wild-type enzyme. Instead, the flexible side chain of WR99210 allowed it to be free from being locked between the residues A16, D54, nicotinamide, and S108 as observed in the structure of the wild-type complex. Therefore it is likely that the steric demand imposed by V16 would not significantly affect the orientation of the drug upon binding to the pfDHFRs with the A16V mutant. This study led to a hypothesis that the resistance to Cyc was owing to a steric clash for Cyc binding as a result of A16V mutation of the pfDHFR.

All the Pyr, Cyc and WR99210 bound to the S108T mutant model with similar orientation as in the wild-type complex, although with some minor differences. The bulkier side chain of the T108 mutant slightly affects the position of the chlorophenyl ring of Pyr and Cyc (Figures 1.1A and 1.1B). Nevertheless, the orientation of the propyloxy substituent of WR99210 was not affected upon binding to pfDHFR with the S108T mutation (Figure 1.1C).

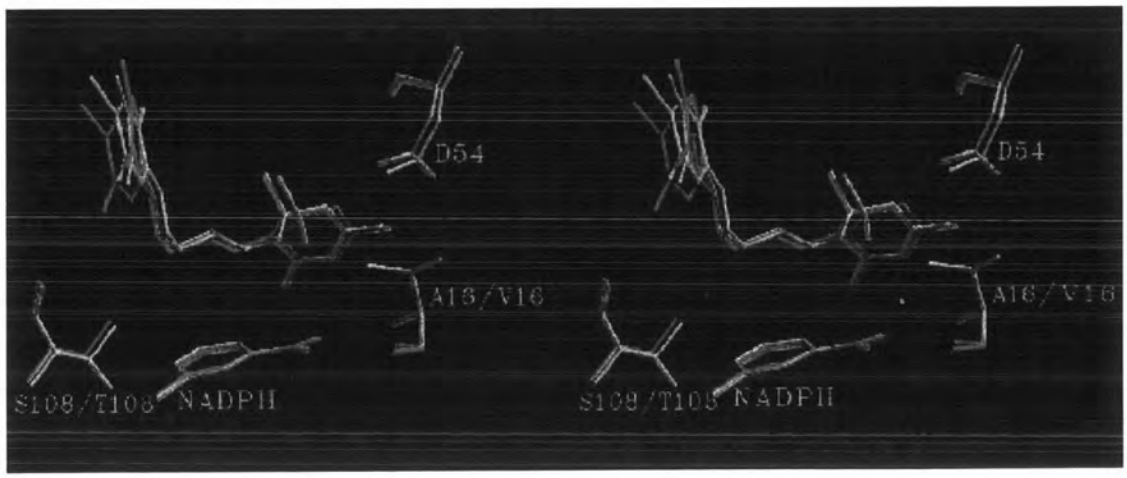
The model showed that the 6-ethyl substituent of Pyr was not in steric conflict with A16V+S108T mutant despite the locking of the inhibitor between residues 16, 54, nicotinamide, and 108. Consequently, the binding of Pyr to the double mutant pfDHFR was not different from the wild-type, A16V, and S108T single mutant enzymes (Figure 1.1A) which is in sharp contrast with Cyc. However, the chlorophenyl ring was further displaced from nicotinamide due to steric conflicts with T108 in the A16V+S108T mutant. While the position of the 4,6-diamino-1,2-dihydro-1,3,5-triazine ring of Cyc was not different to that of the A16V mutant (Figure 1.1B). The binding of WR99210 was similar to Cyc in its 4,6-diamino-1,2-dihydro-1,3,5-triazine moiety yet WR99210 was not locked toward nicotinamide and was also much more flexible. Therefore, the slight displacement of the 4,6-diamino-1,2-dihydro-1,3,5-triazine ring had a consequences for the overall binding in the A16V+S108T mutant (Figure 1.1C).



(A)



(B)



(C)

Figure 1.1 Superimpositions of inhibitors bound to wild-type (yellow), A16V (red), S108T (cyan) and A16V+S108T (white) pfDHFRs. The inhibitors shown were (A) Pyr; (B) Cyc; (C) WR99210.

We had the hypothesis, based from the information above, that the steric effect between one of the C-2 methyl groups of Cyc and the methyl groups of V16 was the major cause that decreased the binding of Cyc with A16V+S108T mutant. It was further proposed that WR99210 was highly effective against both wild-type and A16V+S108T mutant pfDHFR due to its flexible side chain. Therefore, we had the idea to develop new inhibitor of pfDHFR based on the structure of 4,6-diamino-1,2-dihydro-1,3,5-triazine using two approaches. One was decreasing the steric effect of the C-2 methyl groups of Cyc by replacing one methyl group of Cyc with H and the other with alkyl or phenyl group. The other approach was increases the flexibility of the side chain at N-1 position by changing the aromatic to alkyl or alkyloxy group (Scheme 1.10).

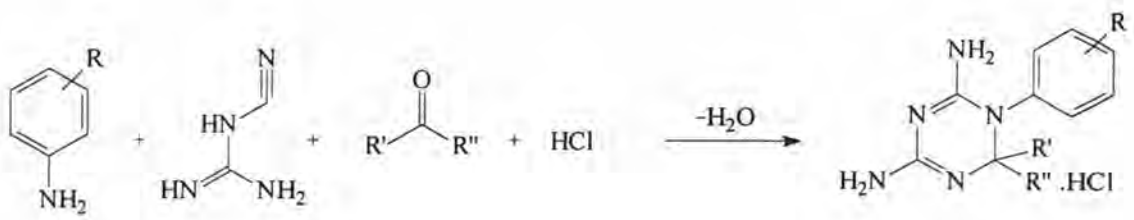
R: alkyl or alkyloxy groups
R': alkyl or aryl groups

Scheme 1.10 Approaches to develop novel inhibitor of pfDHFR the structure of Cyc

The objectives of this research were, first, to synthesize pfDHFR inhibitors which were derivatives of 4,6-diamino-1,2-dihydro-1,3,5-triazines[16,30] by varying the substituents at N-1 and C-2 positions (Schenme 1.10), and, second, to study the relationship between their chemical structures and biological activities against wild-type and A16V+S108T mutant pfDHFRs.

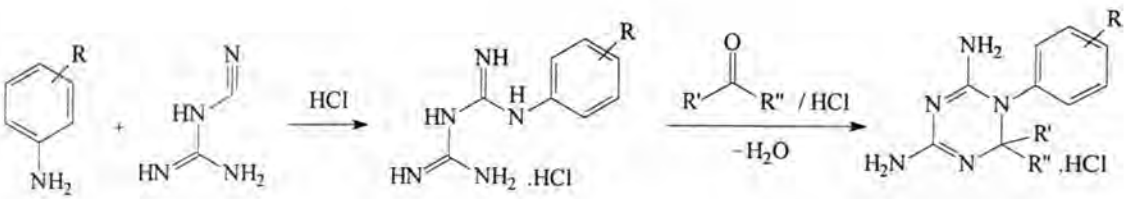
Derivatives of 4,6-diamino-1,2-dihydro-1,3,5-triazine were first synthesized in 1956 by Modest who proposed two methods, namely three-component[31] and two-component synthesis.[32]

The three-component synthesis involves condensation of the arylamine, dicyanodiamide and ketone or aldehyde under acidic condition, with loss of one molecule of water[31] (Scheme 1.11).



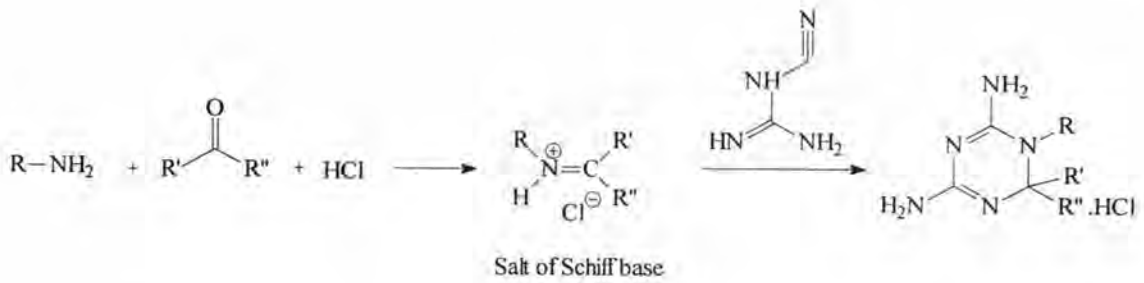
Scheme 1.11 Three-component synthesis

The alternative two-component synthesis involves condensation of an arylbiguanide and a ketone or and aldehyde under acidic condition, also with loss of one molecule of water[32] (Scheme 1.12). The arylbiguanide was prepared from arylamine and dicyanodiamide under acidic condition. It was shown that this condition was more effective than the three-component condensation when the carbonyl compounds were aldehydes.



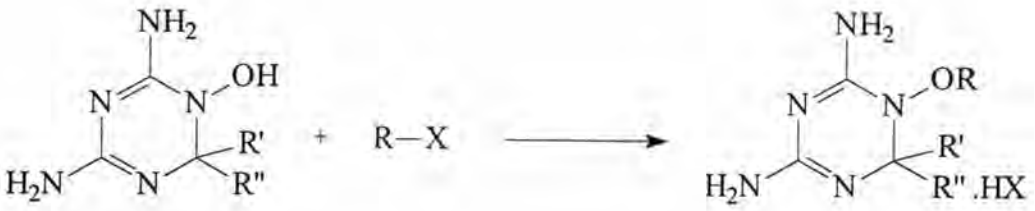
Scheme 1.12 Two-component synthesis

Both methods gave good results only with 1-aryl-4,6-diamino-1,2-dihydro-1,3,5-triazine. In 1964, Newman and Moon found a new method to synthesize derivatives of 4,6-diamino-1,2-dihydro-1,3,5-triazine which are applicable to 1-alkyl-4,6-diamino-1,2-dihydro-1,3,5-triazine as well as 1-aryl-4,6-diamino-1,2-dihydro-1,3,5-triazine. In this reaction, Schiff bases were reacted with dicyanodiamide[33] in the presence of acid catalyst under anhydrous conditions. The Schiff base was generated by reaction of amine with ketone or aldehyde[33] (Scheme 1.13).



Scheme 1.13 The reaction of Schiff bases with dicyanodiamide

Acid catalyzed cyclocondensation of 1-alkyloxybiguanide with carbonyl compound has been reported to produce the corresponding dihydrotriazine.[34] Another method to synthesize 1-alkyloxy-4,6-diamino-1,2-dihydro-1,3,5-triazine involving the alkylation of 1-hydroxy-4,6-diamino-1,2-dihydro-1,3,5-triazine and alkyl halide was also a very effective method to synthesize derivatives of 4,6-diamino-1,2-dihydro-1,3,5-triazines[17,35-38] (Scheme 1.14).



Scheme 1.14 The alkylation of 1-hydroxy-4,6-diamino-1,2-dihydro-1,3,5-triazine and alkyl halide

New derivatives of Cyc and WR99210 will be synthesized using existing or newly developed methods and will be tested against wild-type and A16V+S108T mutant enzymes. The outcome of this research will provide better understanding of the structural basis of the resistance to anti-malarial drugs. This knowledge can lead to a proper design of new inhibitors, which showed better inhibition to A16V+S108T mutant DHFR enzyme as well as wild-type pfDHFR enzyme.