CHAPTER I

INTRODUCTION

Malaria

Malaria, an important infectious disease, remains a major health threat in many areas of the world, especially in tropical and subtropical countries.[1-2] People about 300 to 500 million contract malaria each year. The World Health Organization (WHO) estimates that over one million patients die from malaria each year.[3] Although the number of death caused by malaria has decreased, changing land used, climate and population movement result in the spreading of the disease again.

Human malaria is caused by one of the four species of *Plasmodium* parasites; *Plasmodium falciparum, P. vivax, P. ovale* and *P. malariae*. *P. falciparum* is the most widespread and the most serious, leading cause of morbidity and mortality.[4]

Parasites are transmitted from one person to another by a female anopheline mosquito which was present in almost all countries in the tropics and subtropics.[5] The parasites are carried by the blood to the victim's liver where they live, grow and increase in number of cell. After 9-16 days, they again multiply and begin destroying the red blood cell. Symptoms of malaria illness include headache, back pain, muscle ache, nausea, cough and sometime vomiting, with fever often appeared in most patient. Of all the four species of malarial parasites, only *falciparum* malaria can progress rapidly to obstruct the blood vessels in the brain known as the cerebral stage. Untreated cases can progress to coma, renal failure, liver failure, convulsions and death.

Parasite life cycle

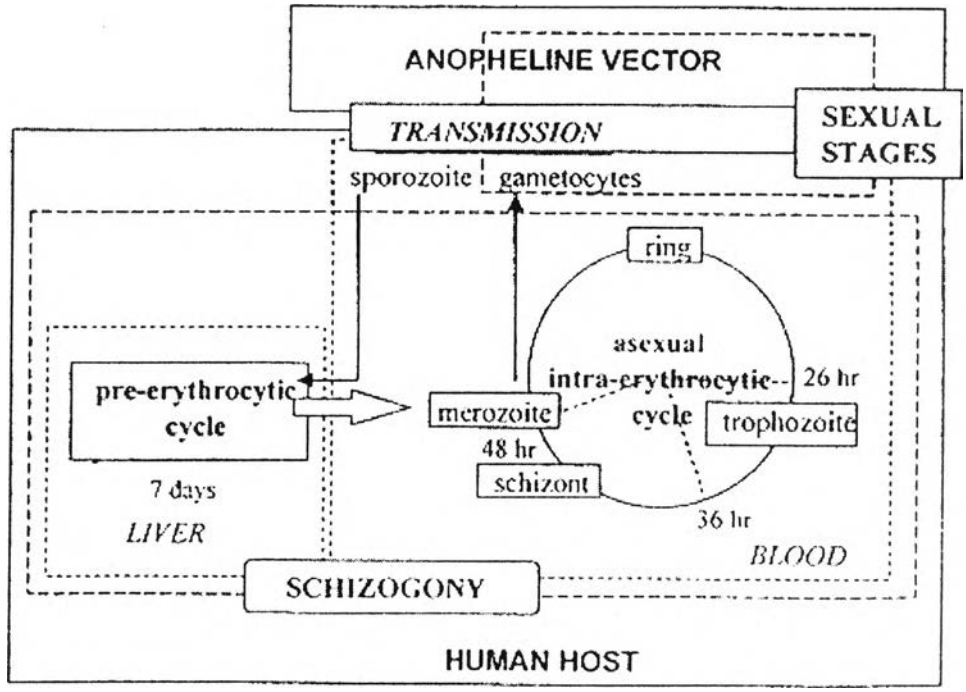


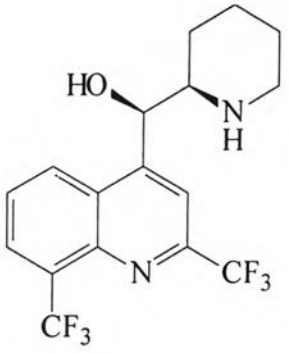
Figure 1.1 Life cycle of malarial parasites

The life cycle of the parasite in human has been described.[4-5] When the human victim was bitten by an infected mosquito, sporozoites move into the blood steam and travel to liver tissue where they invade parenchymal cells. During development and multiplication in the liver known as preerythrocytic stage, there is no sign of infection. After a period of time, merozoites (5,000-4,000 per sporozoite) are released from the liver to reside in the red blood cell in the erythrocytic stage. Attack of the red blood cells by a mereozoite results in the development of the trophozoite stage. The parasite feeds up on the globin, protein portion of hemoglobin and hemozoin, a waste product, accumulates in the host cell cytoplasm. After the parasite undergoes nuclear division, the red blood cell bursts and merozoites and cell debris are released and symptoms of malaria appeared. The merozoites go on to infect more erythrocytes. In addition, a few merozoites become differentiated into male and female which begin sexual reproduction in the mosquito taking a blood meal from the infected human.

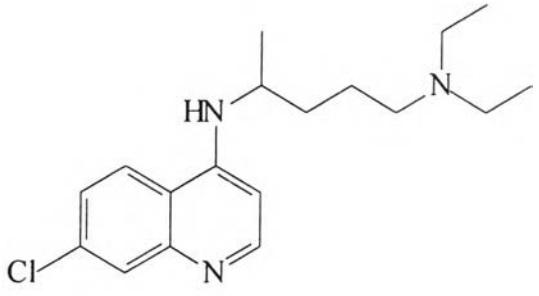
Sporozoites form and then rest in the mosquito saliva. Finally, the infection cycle begins again.

Drugs used in the treatment of malaria

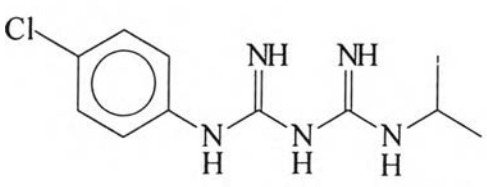
Malaria can normally be treated by antimalarial drugs.[4] The symptoms of illness quickly disappear when the parasites are killed. Many antimalarial agents were developed into drugs such as artemisinin, chloroquine, sulpha, quinine, quinidine, mefloquine, proguanil, pyrimethamine and cycloguanil etc.[6-8]



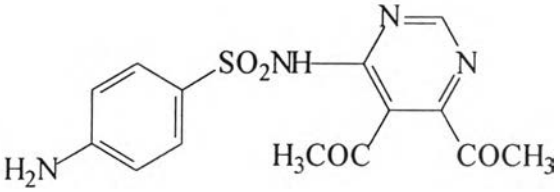
Mefloquine



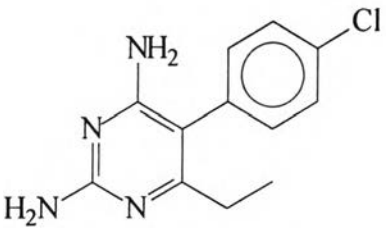
Chloroquine



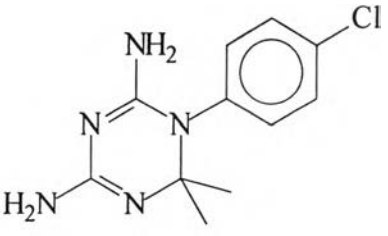
Proguanil



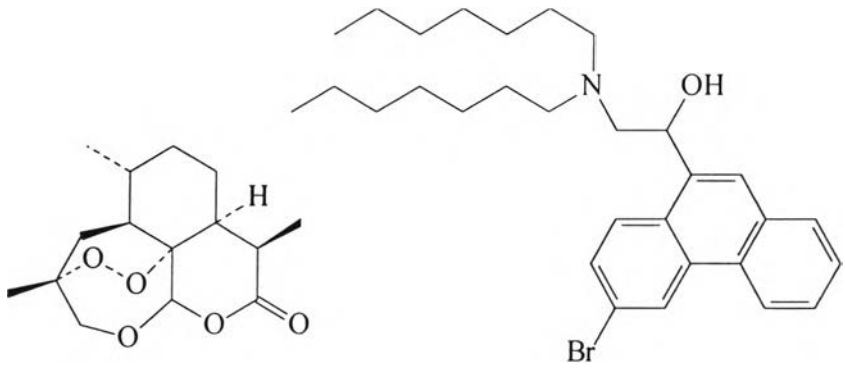
Sulfadoxine



Pyrimethamine



Cycloguanil



Artemisinin WR 33,036

Figure 1.2 Structure of antimalarial drugs

A choice of drugs for treatment in each particular case is determined by multiplicity factors such as the parasite species causing the infection, the susceptibility of the parasite strain to particular antimalarial agents, the facilities and resources available for health care.

Drug resistance

Even though there are many drugs successfully used for malaria treatment in the past, the parasites causing disease have ability to develop of resistance. The phenomenon of drug resistance was wide spread in many areas. A number of drugs, which had been effective in the part, decreases in capability against the parasite. Early in this century, it was noted that some cases of malaria responded much more poorly to quinine, the only drug available at that time. So higher doses of drug had to be taken in order to effect the treatment. Chloroquine, the agent of choice against *P. falciparum* for decades, was fought by the parasite beginning in South America and South-East Asia. The resistance had then spreaded rapidly in Africa. For mefloquine therapy, there was indications that the response to this drugs was remain no longer the case in Thailand. Furthermore, development of drug resistance has also been found with sulfadoxine, pyrimethamine and cycloguanil. The only one currently effective antimalarial drug is artemisinin and derivatives.

At present, the increasing occurrance of drug resistant is still spreading widely. For this reason, a lot of efforts have been made in an attempt to identify new agents which are also effective for the treatment of resistant cases.

DHFR as a target for antimalarial drug development

The study of biology and biochemistry of *Plasmodium sp.* have been of considerable interested because the information from this fields bring about the design of new antimarial agents. Since malaria, a tropical disease, belongs to poor people in the developing world, the development of drug for this disease must be based on cheap and easily synthesized. According to the literature, *P. falciparum* is the most important and most dangerous infection in human. Consequently, most basic metabolic and biochemical process studies were headed for this species.

The initial steps of development was discovering new targets or knowing more about exiting targets. The characteristics of putative targets for antimalarial drug development are displayed in Table 1.1.

Table 1.1 Characteristics of a Putative Target[9]

Essential feature of the parasite life cycle must differ significantly from and analogous process in the host
Lack of alternative pathways that circumvent the target
Elective accessibility to the parasite or accumulation with in the parasite of lead compounds
Low potential for development of resistance
Involvement in a rate-limiting biochemical process
Ability to readily test effects of inhibitors on the target (to validate the target)- test may utilize a lead compound or biochemical or genetic (antisense, transfection) approaches
Presence of a straightforward test system for high through put screening
Existence of know specific inhibitor

The majority of *P. falciparum* life cycle was in the human body. The parasites live in red blood cell and feed on hemoglobin, digesting the protein and releasing the

heme. Hemoglobin digestion proceeds by an ordered metabolic pathway which involves various enzymes.

Folate is an important cofactor in the malarial parasite life cycle. The parasite could either synthesize or take folate from the host.[10] This cofactor, used for development and multiplication of parasite cell, is relevant to many metabolic pathways.

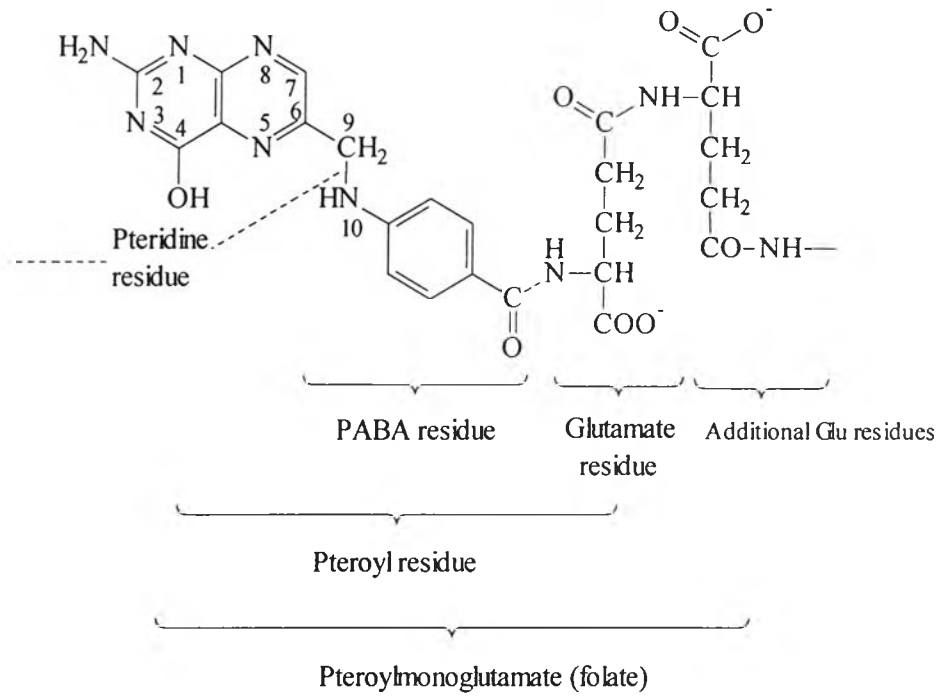


Figure 1.3 Structure of folate

The folate pathway is linked to the purine salvage *via* GTP, with GTP cyclohydrolase, an enzyme used in the *de novo* synthesis pathway.[11] It is also linked to the pyrimidine and amino acid biosynthesis *via* the dTMP synthesis cycle and the methionine synthesis cycle, respectively.

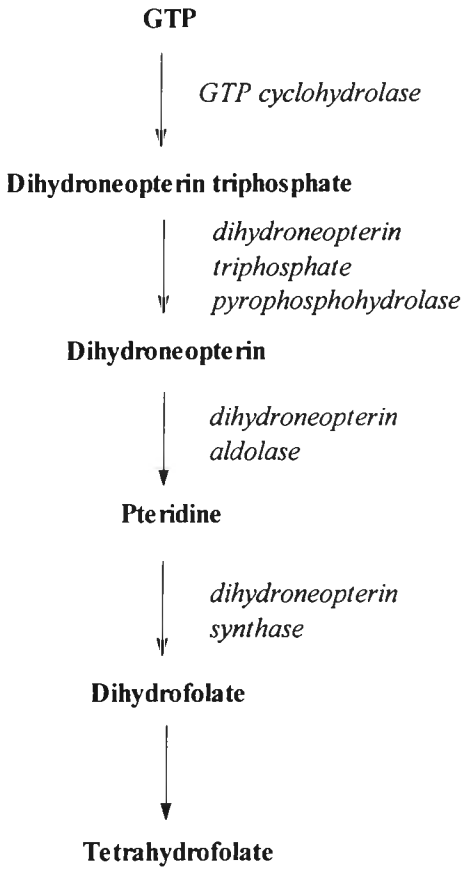


Figure 1.4 Summary of the folate pathway

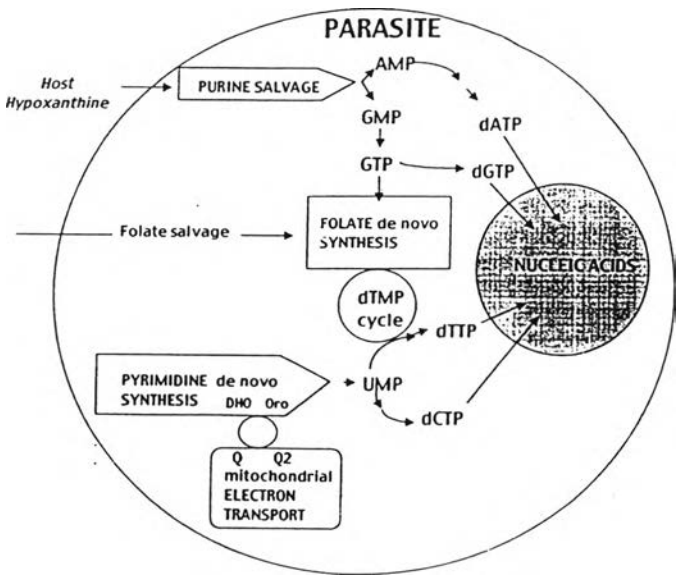


Figure 1.5 Folate in the pathways of nucleic acid

Table 1.2 Metabolism of Nucleic Acids in Humans and Plasmodia[9]

Pathway	*Plasmodium*	Mammal
Pyrimidines	Synthesizes pyrimidines *de novo*; can not salvage bases/ nucleotides	Can either synthesize or salvage pyrimidine nucleotides
Purines	No *de novo* synthesis; relies on host-derived hypoxanthine as source of purine precursors	Can either synthesize or salvage purine nucleotides-hypoxanthine product
Folate cofactors	Can either synthesize or salvage folate precursors	No *de novo* synthesis; rely on external sources

There were many enzymes working coorperatively in the malarial metabolic and biochemical processes. Dihydrofolate reductase (DHFR), is one of the key enzyme which is responsible for the reduction of dihydrofolate (FH_2) to tetrahydrofolate (FH_4),[12] a necessary cofactor for biosynthesis of thymidylate, purine, nucleotides and amino acid (Figure 1.6).[9] If the DHFR is not available, the malarial life cycle would be cut off. This caused the death of the malarial parasites.

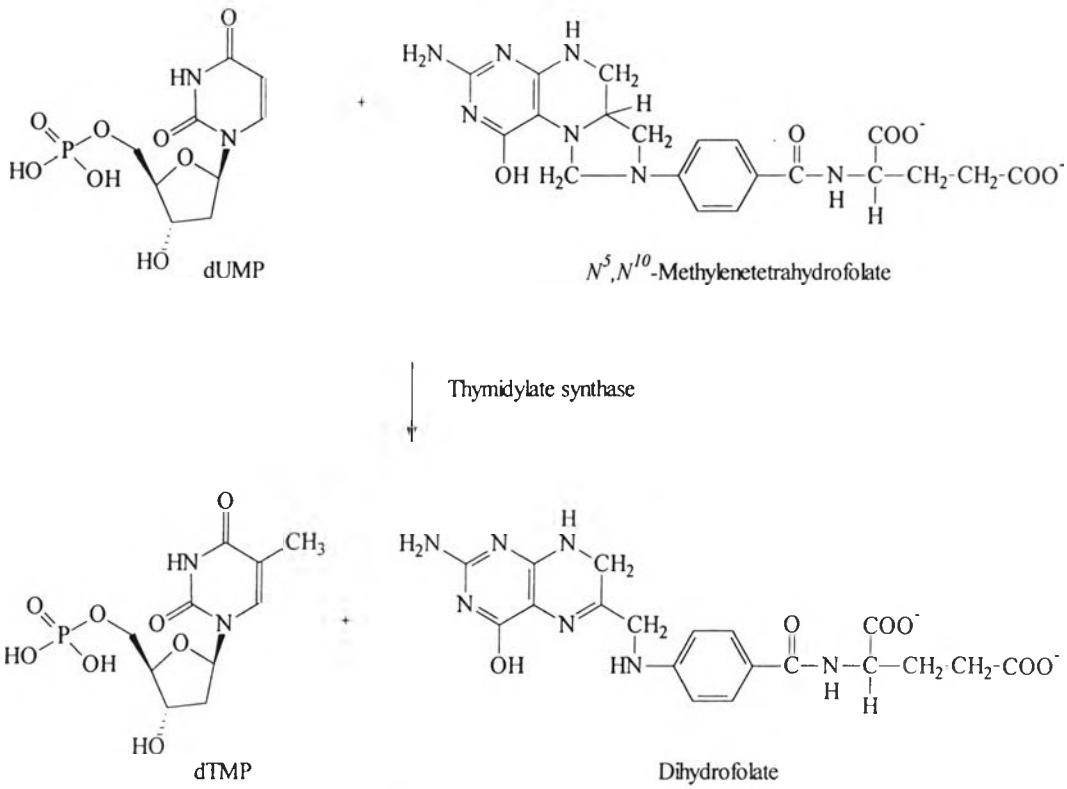


Figure 1.6 Thymidylate synthesis[13]

Many antimalarial agents which have been successfully developed to drugs such as pyrimethamine, cycloguanil act against parasite by inhibition of enzyme DHFR. Because of the development of drug resistance, pyrimethamine and cycloguanil lost effective in curing nowadays. Parasite resist this agents by changing some amino acids in active site of DHFR to different ones known as mutation.[14-23] However, this compound and its analogue are still of interest in family approach to antimalarial drug design. It was hoped that a better understanding of mechanisms of resistant to this class of compounds might lead to development of better inhibitor in this family which are effective not only to the wild-type, but also to the resistant malaria parasites as well.

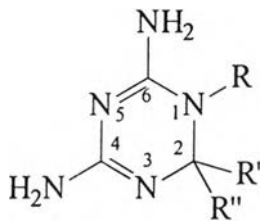
Indentification of DHFR

Mutation of DHFR,[14-23] a key molecular target, had been identified. From the literature,[24] Molecular modeling studies of the interaction between antimalarial antifolates and DHFR both wild type and A16V108T-mutant caused by mutation at amino acid residues 16 and 108 resulted in the prediction that reducing steric effect at

C_2 will improve binding to this mutant enzyme. The experiment data in Table 1.2 displayed the binding affinity (K_i) values for the cycloguanil and its derivatives which are in good agreement with the prediction.

Cycloguanil **(I)** and its bromoderivative **(II)** bind well with wild type DHFR but a great elevation of the K_i to A16VS108T mutant was observed. When one of the methyl group at C_2 was replaced by H as in compound **(III)**, the interaction between this compound and A16VS108T was about 50 times better compared to compound **(II)** and cycloguanil. The same result was observed in the case of compound **(IV)** and **(V)**. The K_i-mut/K_i-wild ratio greatly improved (~100 times) when the compound had only one substituent group at C_2.

Table 1.3 K_i values for the cycloguanil and derivatives compared with their 2-mono-methyl derivatives[24]



compound	1-R	2-R'	2-R''	K_i-wt* (nM)	K_i-mut** (nm)	K_i-mut/Ki-wt
I	4-ClC_6H_4	Me	Me	1.5 ± 0.3	1,314 ± 165	876
II	4-BrC_6H_4	Me	Me	1.1 ± 0.2	1947 ± 366	1771
III	4-BrC_6H_4	H	Me	5.7 ± 0.5	202 ± 17	35
IV	4-$CH_3C_6H_4$	Me	Me	1.8 ± 0.2	1,584 ± 210	880
V	4-$CH_3C_6H_4$	H	Me	23.4 ± 1.9	186 ± 22	8

* wild-type pfDHFR

** pfDHFR (A16VS108T)

The information from Table 1.2 suggested that resistance to cycloguanil and its derivatives in the A16VS108T mutant DHFR occurred due to steric conflict imposed by a bulkier side chain of V16 as compared to A16 in the wild-type enzyme. The steric clash between the V16 side chain of A16V mutant DHFR and cycloguanil according to a recent model by Rastelli[24] was shown in Figure 1.7.

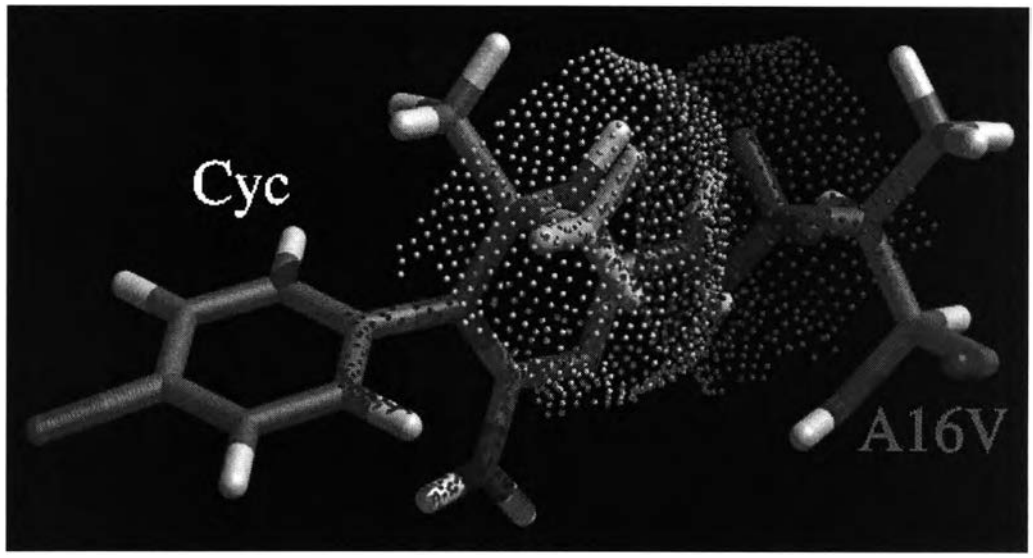


Figure 1.7 Model of cycloguanil and analogues bound to wild type and A16VS108T mutant DHFR

The molecular modeling study revealed specific binding of DHFR with cycloguanil and its analogues. For the interaction between the cycloguanil derivatives and A16VS108T, it was predicted that better inhibitor would be made by arrangement for avoiding or decreasing the steric effect by turning the smaller substitued groups at C_2 to a bulkier side chain of V16.

Since there are two enantiomers of dihydrotriazine derivatives which are unsymmetrically substituted at C_2, the next question would be which stereoisomer binds better to the wild-type and A16VS108T mutant DHFR. The model predicted that binding of one enantiomer, which was in C_2-*S* configuration, to DHFR should be better than the other one which possesses the C_2-*R*-configuration.[24] proving this experimentally will lead us to better understanding on the molecular basis of resistance. Moreover, this result might bring news insights in development of new antimalarial molecules. For this reason, resolution of cycloguanil derivatives in order to study their biological activities is the objective for this research.

Resolution of 4, 6-diamino-1, 2-dihydro-1, 3, 5 triazine

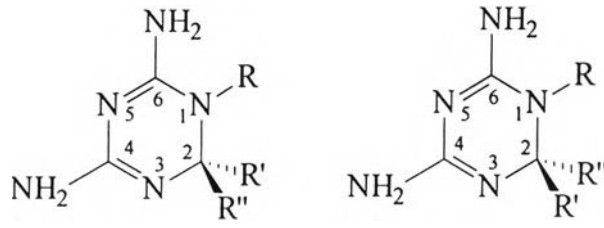


Figure 1.8 Structure of enantiomer of 4,6-diamino-1,2-dihydro-1,3,5 triazine

Cycloguanil and its derivatives, which are of 4,6-diamino-1,2-dihydro-1,3,5 triazine, family were synthesized from achiral precursors. Racemates will result when the substitution of R′ and R″ are not identical. As enantiomers possess identical physical properties, separation of mixture of enantiomers could not be accomplished by conventional methods such as crystallization, distillation or chromatography.

There have been several methods used to separate enantiomers.[24-27] These are collectively known as resolution which includes:

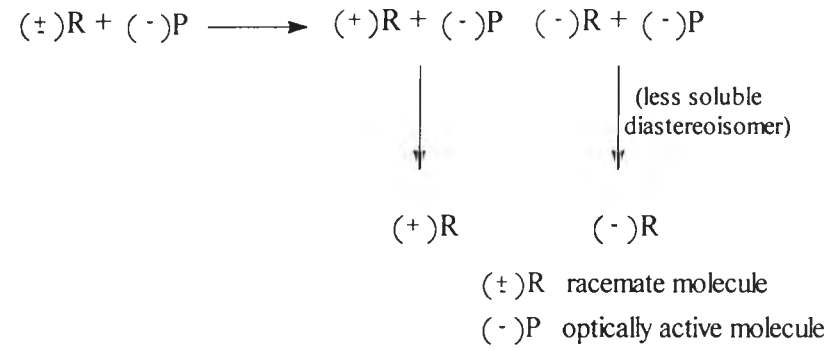
- resolution *via* formation of diasterioisomeric salts or complexes
- resolution *via* formation of diastereoismeric covalent compounds
- resolution by means of chromatography on a chiral phase
- kinetic resolution with enzyme
- enantioselective synthesis (not resolution)

In this thesis, two possible separation techniques were proposed for resolution 4,6-diamino-1,2-dihydro-1,3,5-triazine *via* diasteroisomer and resolution by means of chromatography on a chiral phase.

Resolution *via* diastereoisomers

The principle of this method involve introducing a new chiral centre into the racemate molecule by with combining it with an optically active compound or chiral molecule,[28-30] which may form salts, complexes or covalently attached. The products would be diastereoisomers which could be separated by crystallization, distillation or chromatographically. This is the most common technique employed in resolution and

almost all chiral compound have been successfully resolved in this way.



Resolution of a few dihydrotriazine derivatives by this approach have been made in the past, but was reported to be unsuccessful.[31] In this study, a wider range of conditions will be tested.

Resolution by means of chromatography on a chiral phase[27]

This technique involves the differential formation of diastereomeric complexes by adsorption of racemates on a chiral solid phase. The success in resolution depended on the difference in adsorption ability in each of enantiomers. The isomer which was adsorped better passes through the column more slowly while the other passes more rapidly. There were two kinds of chiral phases were applied in chromatography separation. One was normal chiral phase containing nonpolar optically active molecule coated on the support. The enantiomer which less polar diastereomer was formed or absorbed with these solid phase better than the other. So it would be the last enantiomer passing through the column. In contrast, the optically active molecule which imposed polarity property on solid support known as chiral reverse phases separate the racemate by adsorbing the enantiomer which was more polar than the other. There, nonpolar enantiomer would pass the column first while the other one was followed. Resolution by means of chromatography on a chiral phase had also been applied to gas chromatography and liquid chromatography on optically active ion exchangers.

After the separation was successful, the absolute configuration of the pure enantiomers will be studied by X-ray crystallography and/or circular dichroism spectroscopy. The resolved enantiomers will then be tested against the DHFR enzyme in order to validate the predicted model.