# ANTIFOLATE RESISTANCE MUTATION AND PROGUANIL SUSCEPTIBILITY AMONG *PLASMODIUM FALCIPARUM* ISOLATES IN THAI-CAMBODIA BORDER

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**ABSTRACT:** Malaria is still one of the major public health problems in Thailand. Malaria parasite, *Plasmodium falciparum*, has been found to resist to most available antimalarial drugs. Although the ACTs base combination is still effective against falciparum infection, few resistant cases were reported. Malarone, a combination of atovaquone and proguanil, had been introduced for prophylaxis and treatment in Trat and Chanthaburi Provinces, 2009 – 2011, to prolong the life span of the ACTs base combination therapy. The purpose of this study is to assess the point mutation of *in vitro* susceptibility and *dhfr* gene against proguanil before and after the administration of Malarone. A total of 37 blood samples collected from patients attending malaria clinic at Trat and Chanthaburi Province during 1993 to 2011 were analysed. The results revealed four mutations at $51^{N/I}$, $59^{C/R}$, $108^{S/NT}$ and $164^{I/L}$ residues in the *dhfr* gene. All samples had at least three point mutations and moderately resistant against proguanil with MIC ranging from $10^{-5}$-$5x10^{-5}$ M. These mutations were also maintained in falciparum samples collected from the same areas prior to and during Malarone usage. The number and position of point mutations were correlated with proguanil susceptibility. Although the proguanil alone should not be recommended for falciparum malaria prophylaxis and treatment in this region, proguanil and atovaquone combination, with their synergistic effects, may effective against falciparum treatment.

**Keywords:** *Plasmodium falciparum*, *dihydrofolate reductase* gene, Malarone, proguanil susceptibility

## INTRODUCTION

At present, multidrug resistant malaria parasites, *P. falciparum*, can be found in many parts of the world. In Thailand, the border area between Thailand and Combodia has been the center of multidrug resistant malaria [1, 2]. The parasites became sequentially resistant against chloroquine, sulfadoxine-pyrimethamine and mefloquine [3-5]. As a result, ACTs base combination has been used in this area. The combination proved to be effective, but few resistant cases were reported. A new drug, Malarone, a proguanil and atovaquone combination had been introduced for prophylaxis and treatment in Trat Province to prolong the life span of the ACTs base combination therapy during 2009-2011.

Proguanil is an antifolate drug. It acts as a dihydrofolate reductase (DHFR) inhibitor, so the pyrimidine synthesis is inhibited. The point mutations in the *dhfr* gene are the molecular basis of cycloguanil resistance in falciparum malaria, especially a 108 amino acid residue. Other mutations at 16, 51, 59 and 164 residues were also reported. These mutations were proposed to correlate with proguanil resistance [6].

Drug susceptibility of *P. falciparum* can be performed through several approaches, microscopic assay, isotopic assay and histidine – rich protein II and lactate dehydrogenase detection. The microscopic assay is the only method which directly observes the living and abnormal parasites in thin blood smear stained with Giemsa. This technique has been considered as a standard method for drug susceptibility test.

In this study, *in vitro* susceptibility against proguanil and the point mutation of *dhfr* gene, before and during the administration of Malarone, were assessed. These baseline data is importance for following up the drug resistance problem in this area.

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**Table 1** Thirty seven isolates of *P. falciparum* were collected during 1990-2011

| Isolates | Collected years | Isolates | Collected years |
|---|---|---|---|
| TD3 | 1990 | TD541, TD542, TD544, TD545, TD547 | 2006 |
| BR17,BR26, BR102 | 1991 | TD550 | 2007 |
| TD373, TD371 | 1992 | TD554, TD556 | 2008 |
| TD498, TD500 | 1993 | CH1, CH2, CH3, CH4, CH6, CH7 | 2009 |
| TD506 | 2003 | TD559, TD560, TD68, TD73, TD76, TD77, CH8, CH9 | 2010 |
| TD530, TD533 | 2005 | TD79, TD80,TD81,TD82 | 2011 |

## MATERIALS AND METHODS

### Malaria sample

All 37 parasite samples were obtained from Malaria Research Program, Chulalongkorn University, Thailand and Malaria Clinic. Parasite samples were collected from Trat and Chanthaburi provinces, Thailand during 1990-2011 (Table 1). Some of these samples were collected in filter papers from which their genomic DNA were extracted and sequenced.

### Culture falciparum parasite

*P. falciparum* isolates were cultured by the candle-jar method [7]. The washed parasite pellet from a cryopreserved sample was added into culture dishes contained 3 ml of complete RPMI media and uninfected red blood cell. The parasite culture was placed in a dessicator with lit candle and was incubated at 37°C. The complete medium and fresh uninfected red blood cells were added every 4 day. To observe the parasite, thin blood films were prepared and stained with the Giemsa stain. Percent parasitaemia was evaluated with a compound microscope at 1000x magnification.

### *In vitro* drug sensitivity assay

Drug susceptibility of *P. falciparum* isolates to proguanil hydrochloride was determined using minimum inhibitory concentration test (MIC) [8, 9]. Prior to the test, the parasite was synchronized with D-sorbitol to select for the ring stage. Briefly, the parasite (more than 5% parasitaemia) were harvested and pooled in a microfuge tube. The tube was spun at 1,500 rpm for 10 min at room temperature. After the supernatant was removed, 5 times of pack cell volume of 5% D-sorbitol was added and gently mixed. This parasite-sorbitol mixture was incubated at room temperature for 10 min. An excess volume of complete medium was added and the tube was centrifuged at 1,500 rpm for 10 min at room temperature. After, the supernatant was removed, the parasite pellet was washed with complete medium. For the drug susceptibility test, the synchronized parasite was diluted with uninfected red blood cell to 0.3 – 0.5 percent parasitaemia. A 10 µl of parasite was dispensed into each wells of 96 micro-well plate containing 100 µl of RPMI complete medium containing antimalaria drug at various concentration, $5x10^{-6}$, $10^{-5}$, $2.5x10^{-5}$, $5x10^{-5}$ and $10^{-4}$ M respectively. The test was performed in triplicate with RPMI complete medium as a control.

During the experiment, the culture medium (with or without drug) were changed daily. After 72 hour, thin blood films were prepared from each wells and stained with the Giemsa stain. The presence of parasites was observed under a compound microscope. The minimum drug concentration which killed all or nearly all parasites was reported as the minimum inhibitory concentration (MIC) for each parasite isolates.

### Genomic DNA extraction

Genomic DNA of malaria parasites were extracted by phenol-chloroform method as described by Snounou [10]. Some of samples were extracted from filter papers as described by [11]. The DNA stock solutions were preserved at -20°C until use.

### Polymerase chain reaction [12]

The *dhfr* genes were amplified by semi nested-PCR method. The concentration of PCR reagents in the mixture were adjusted to 1X *Taq* DNA polymerase buffer, 2 mM magnesium chloride, 0.25 mM deoxynucleotide triphosphate, 0.25 mM primer and 2.5 unit/µl *Taq* DNA polymerase together with 1 µl of diluted DNA template in 20 µl reaction volume. The temperature cycle were set as follows: denaturation (95°C, 30 sec) annealing (58°C, 30 sec) and extension (72°C, 1.5 min) for 30 cycles using the *Veriti* (TM) 96-Well Thermal Cycler (Applied Biosystems). The forward and reverse primers were DHFR1F (5′ATGATGGAACAAGTCTGC GACGTTT 3′), DHFR857R (5′ TCTTCATCATCATCATCATTTTCA 3′) and DHFR1449R (5′AAGGAGGTAATGCCATTTGG 3′).

**Table 2** Drug susceptibility values against proguanil of fifteen parasites were determined in Molar unit as MIC, minimum inhibitory concentration.

| ***P. falciparum*** **isolates/clone** | **Proguanil (MIC)** | **Mutations (amino acid residues)** | | | | |
|---|---|---|---|---|---|---|
| | | **[1]16$^{A/V}$** | **51$^{N/I}$** | **59$^{C/R}$** | **108$^{S/NT}$** | **164$^{I/L}$** |
| [2]K1CB1 | $10^{-4}$ | A | N | R | N | I |
| TD498 | $5x10^{-5}$ | A | I | R | N | I |
| TD500 | $5x10^{-5}$ | A | I | R | N | I |
| TD506 | $5x10^{-5}$ | A | I | R | N | I |
| TD547 | $5x10^{-5}$ | A | I | R | N | I |
| TD3 | $5x10^{-5}$ | A | I | R | N | L |
| TD559 | $5x10^{-5}$ | A | I | R | N | L |
| TD530 | $2.5x10^{-5}$ | A | I | R | N | L |
| TD533 | $2.5x10^{-5}$ | A | I | R | N | L |
| TD541 | $2.5x10^{-5}$ | A | I | R | N | L |
| TD554 | $2.5x10^{-5}$ | A | I | R | N | L |
| TD556 | $2.5x10^{-5}$ | A | I | R | N | L |
| TD545 | $2.5x10^{-5}$ | A | I | R | N | I |
| TD550 | $2.5x10^{-5}$ | A | I | R | N | I |
| TD542 | $10^{-5}$ | A | I | R | N | L |
| TD544 | $10^{-5}$ | A | I | R | N | L |
| TD373 | [3]ND | A | I | R | N | L |
| TD371 | ND | A | I | R | N | L |
| TD560 | ND | A | I | R | N | L |
| TD68 | ND | ND | I | R | N | L |
| TD73 | ND | ND | I | R | N | L |
| TD76 | ND | A | I | R | N | L |
| TD77 | ND | ND | I | R | N | I |
| TD79 | ND | ND | I | R | N | L |
| TD80 | ND | ND | I | R | N | L |
| TD81 | ND | ND | I | R | N | L |
| TD82 | ND | ND | I | R | N | L |
| CH1 | ND | A | I | R | N | I |
| CH2 | ND | A | I | R | N | L |
| CH3 | ND | A | I | R | N | L |
| CH4 | ND | A | I | R | N | I |
| CH6 | ND | A | I | R | N | I |
| CH7 | ND | ND | I | R | N | L |
| CH8 | ND | ND | I | R | N | L |
| CH9 | ND | A | I | R | N | L |
| BR 17 | ND | A | I | R | N | L |
| BR26 | ND | A | I | R | N | L |
| BR102 | ND | A | I | R | N | L |

[1]The mutation positions were reported as [amino acid residue $^{\text{wild type/mutated amino acids}}$]. Wild type amino acid residues were showed in gray.
[2]K1CB1 is a proguanil-resistant clone, used as a control.
[3]ND = No Data.

**Detection of PCR product**

The amplified products were detected with agarose gel electrophoresis [13]. Graphic information was captured by the Autochemi$^{TM}$ system (UVP, LLC).

**DNA sequencing and sequence alignment**

The PCR products were purified and sequenced by Bio Basic Canada Inc. The sequences from both strands of PCR products were aligned with Bioedit program. The sequences of each isolate were deduced from the matched sequence of both strands. Finally, all sequences were compared and analyzed for gene mutations.

## RESULTS

From thirty seven parasite isolates, 4 mutations were found at 51, 59, 108 and 164 amino acid residues of the dihydrofolate reductase gene (Table 2). All isolates had at least three mutations, isoleucine, arginine and asparagine amino acids at residues 51, 59 and 108, respectively. Both wild type amino acid, isoleucine, and mutated amino acid, leucine, were found at 164 residue. In

comparison to all isolates from Trat and Chanthaburi provinces, the K1CB1 clone, a proguanil-resistant clone, had a unique wild type amino acid, asparagine at 51 residues while 59 and 108 residues were mutated. In comparison, the *dhfr* gene mutations remain the same prior to and after the administration of Malarone (Table 2).

Fifteen *Plasmodium falciparum* isolates from Trad province showed different levels of MIC value against proguanil. The MIC values were divided into three groups, $5x10^{-5}$, $2.5x10^{-5}$ and $10^{-5}$ M. There were 6 and 7 isolates exhibited MIC at $5x10^{-5}$ and $2.5x10^{-5}$ respectively (Table 2). Only two isolates, TD542 and TD544, showed the lowest MIC, $10^{-5}$ M, in this test.

## DISCUSSIONS

The MIC values of all isolates were lower than the MIC of the K1CB1, $10^{-4}$ M. Three MIC values, $5x10^{-5}$ and $2.5x10^{-5}$ M can be found in parasite samples collected from 1990-2010, except $10^{-5}$ M which was found only in two samples collected in 2006. It must be noted that more samples from 2006 were included in this experiment. This may increase the chance for different MIC to be found as all three MIC values can be detected in the samples collected from 2006.

Previous reports suggested that point mutations in *dhfr* gene were correlated with susceptibility against proguanil. The level of antifolate drug susceptibility was depended on numbers and positions of point mutations. Five mutations at amino acid residue 16, 51, 59, 108 and 164 had been reported [6, 14]. In this experiment, four mutated residues were detected at residues 51, 59 and 108 in all samples. Two forms of amino acids, wild type and mutant, were detected at residue 164. The mutated amino acid form, at 51 and 164 residues, appeared to lower the MIC value compared to the wild type amino acid. However, TD545 and TD550, contained wild type residue at 164, showed two times lower MIC than those isolates which have the same sequence, TD498, TD500, TD506 and TD547. On the other hand, isolates which has the mutated amino acid at 164 residue resisted to proguanil at different level, e.g. TD559 and TD544. It suggested that other genetic factors located outside the *dhfr* gene may contribute to proguanil and/or other antifolate susceptibility [15].

Pyrimethamine, an antifolate drug, and its combination were tested and reported as an effective antimalaria drug in Thailand during 1970s [16-20]. About ten years later, resistant isolates against pyrimethamine combination drug had been found. Amino acid changes in DHFR enzyme had been reported to associate with resistance against antifolate drugs (both pyrimethamine and proguanil) [21-24]. Point mutations in the *dhfr* gene of parasite samples collected during 1990 – 2011, prior to and during Malarone usage were similar to those reported nearly 40 years ago. This suggested that limited amino acid changes were allowed to maintain the DHFR enzyme function.

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