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Characterization of *Plasmodium knowlesi* dihydrofolate reductase-thymidylate synthase and sensitivity to antifolates

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ABSTRACT

Malaria caused by an infection of *Plasmodium knowlesi* can result in high parasitemia and deaths. Therefore, effective and prompt treatment is necessary to reduce morbidity and mortality. The study aims to characterize *P. knowlesi* dihydrofolate reductase-thymidylate synthase enzyme (*Pk*DHFR-TS) and its sensitivity to antifolates. The putative *Pkdhfr* gene was PCR amplified from field isolates collected from the Southern Thailand. Molecular analysis showed 11 polymorphisms in the *dhfr* domain of the bifunctional *dhfr-ts* gene. Of these, 1 polymorphism was a non-synonymous substitution (R34L) that had previously been reported but not associated with antifolate resistance. The recombinant *Pk*DHFR-TS enzyme was found to be sensitive to standard antifolates—pyrimethamine and cycloguanil—as well as P218, a registered candidate drug currently first in human clinical trial. Results suggest that antifolates class of compounds should be effective against *P. knowlesi* infection.

1. Introduction

Plasmodium is an Apicomplexan protozoan that causes malaria infection. Presently, fives species -*Plasmodium falciparum (Pf)*, *P. vivax (Pv)*, *P. ovale (Po)*, *P. malariae (Pm)*, and *P. knowlesi (Pk)* - are known to cause diseases in human. The long-tailed and pig-tailed macaques were identified as the natural reservoir for *P. knowlesi* until 1965 when the first naturally acquired infection in man was reported [1, 2]. The reported numbers of infected cases by *P. knowlesi* are small compared to those of *P. falciparum* and *P. vivax*. The burden caused by this parasite may have been overlooked as it is often misdiagnosed as *P. malariae* by microscopic examination [3, 4]. In contrast to patient infected with *P. malariae*, *P. knowlesi* infection usually shows high parasitemia in association with severe symptoms and death [3].

Recent studies have shown wide distribution of *P. knowlesi* in different regions of Malaysia and countries in Southeast Asia [5–7]. The first confirmed case of *P. knowlesi* in Thailand was reported in 2004 in a patient who had traveled to a southern province neighboring with Myanmar [5]. In a large scale study in 5 provinces in Thailand bordering either Myanmar, Cambodia or Malaysia, approximate 0.57% microscope-diagnosed malaria cases were later identified to be infected or co-infected with *P. knowlesi* by PCR analysis [8]. Political unrest in the 3 most southern provinces of Thailand bordering with Malaysia has disrupted routine malaria control program, making accurate diagnosis and treatment of malaria including diagnosis of malaria and *P. knowlesi* very challenging, and led to an increase in the overall malaria cases [8, 9]. In addition, an increasing popularity of eco-tourism in this region potentially amplifies the threat by *P. knowlesi*. Recent examples of *P. knowlesi* cases have been reported among travelers to the Thailand-Malaysia border [10, 11].

While chloroquine treatment for *P. falciparum* has been ineffective due to widespread resistance, the treatment is still relatively effective against non-*falciparum* malaria including *P. knowlesi* [12]. Similarly, antifolate resistance soon developed in *P. falciparum* after large scale deployment of sulfadoxine-pyrimethamine (SP). Although, SP is not used as treatment for non-*falciparum* malaria, drug exposure during mixed infection with *P. falciparum* could potentially induce selection pressure for antifolate resistance.

Mechanisms of inhibition and resistance to antifolate against the enzyme target, dihydrofolate reductase of the bifunctional dihydrofolate reductase-thymidylate synthase (DHFR-TS) enzyme, have been well studied and have significantly contributed toward research

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for new compounds to overcome resistance [13]. Structural comparison of *Pf*DHFR and *Pv*DHFR wildtype enzymes with corresponding resistant enzymes in complexes with pyrimethamine have confirmed the key amino acids responsible for drug inhibition and drug resistance [14, 15]. Interestingly, there have been reports in Africa that after a prolonged removal of drug selection pressure, the antifolate sensitive DHFR genotypes can reemerge over time [16, 17]. This may be possible because antifolate resistant phenotypes also come at a fitness cost to the malaria parasites [18–20]. To date, the *Pkdhfr-ts* gene has been reported but its functional confirmation and enzyme properties have not been reported. To better understand *Pk*DHFR enzyme properties and its sensitivity to antifolate compounds, here we described genetic and biochemical analysis of the *Pk*DHFR-TS properties.

2. Materials and methods

2.1. Genomic DNA preparation from dried blood spots (DBS)

A 3 mm diameter spot was punched out from 2 DBS samples of patients diagnosed with *P. knowlesi* infection and dropped into the bottom of one well on a 96-well plate. Genomic DNA (gDNA) extraction from DBS was conducted using QIAamp® 96 DNA Blood Kit according to the manufacturer's protocol (Germantown, MD, USA). Extracted gDNA sample was transferred into new microfuge tube and stored at −20 °C until ready to use for PCR. The study protocol was approved by the Institution Review Board Committee of Royal Thai Army Medical Department (Protocol number S010h/50), overseeing the procedure performed by staffs of Phramongkutklao College of Medicine.

2.2. Cloning of Pkdhfr-ts gene

The full-length *Pkdhfr-ts* gene was amplified from PKNY145CSP isolate using nested primer pairs that were designed based on the reference sequence (PKNH_0509600, www.plasmodb.org). For the first round of PCR, a 25 μL PCR contained 50 ng of gDNA purified from DBS, 0.4 μM each of *Pkdhfr*UTRFwd (5′-CACCTGCACATACATCGTCAC-3′) and *Pkdhfr*UTRRev (5′-GCTAGCTAGCTAGCTAACTAG-3′), 1× Phusion HF buffer, 0.2 mM dNTPs, and 0.5 unit of Phusion® High-Fidelity DNA polymerase (NEB, Ipswich, MA, USA), and subjected to the PCR conditions; 30 s at 98 °C for denaturing step, 30 cycles of 10 s at 98 °C, 30 s at 55 °C, and 1 min 30 s at 72 °C for amplification step, 5 min at 72 °C final extension step. For the nested PCR reaction, 1 μL of the product from first round of PCR was added to second PCR reaction containing 0.4 μM each of *Pkdhfr*NdeIATGFwd (5′-GGCATATGGAAGACCTTTCAG AGG-3′) and *Pkdhfr*TAGHind3Rev (5′-CTTTCGAACTAGGCGGCCATC TCC-3′), 1× Phusion HF buffer, 0.2 mM dNTPs, and 0.5 unit of Phusion® High-Fidelity DNA polymerase in a 50 μL reaction. Nested PCR reaction was performed for 30 s at 98 °C for denaturing step, 30 cycles of 10 s at 98 °C, 30 s at 58 °C, and 1 min at 72 °C for amplification step, 5 min at 72 °C final extension step. The final product included the 1881 bp full length *Pkdhfr-ts* gene flanked at the 5′- and 3′-end with *Nde*I and *Hind*III restriction sites, respectively. The final PCR product was purified and cloned into pET17b vector at *Nde*I and *Hind*III sites and the resulting construct was named pET17b*Pk*DHFR-TS. The sequence of *Pkdhfrts* in the construct was analyzed by 1st BASE (Selangor Darul Ehsan, Malaysia).

2.3. Bacterial complementation

To obtain bacterial transformants, standard heat shock method was used. Briefly, competent *E. coli* cells (BL21(DE3) or χ 2913recA-DE3) and approximately 100 ng of plasmid of interests were allowed to incubate on ice, and then heat shocked by placing at 42 °C for 45 s, and then placed back in ice. LB broth was added to the sample and the transformed cells were incubated for 37 °C for 30 min with shaking. Transformants were picked and cultured in LB broth supplemented with 100 μg/mL ampicillin at 37 °C.

To determine malaria DHFR activity in bacterial complement system, the experiments were carried out as described previously [21, 22]. In brief, BL21(DE3) harboring pET17b*Pk*DHFR-TS was spotted on a minimum media (MM) agar supplemented with 100 μg/mL ampicillin with or without 2 μM trimethoprim. For the TS activity, χ 2913recA-DE3 (ΔthyA572, recA56) [23] carrying pET17b*Pk*DHFR-TS was spotted on a MM agar supplemented with 100 μg/mL ampicillin with or without 50 μg/mL thymidine. In both experiments, cells carrying vector alone (pET17b) and vector with *Pv*DHFR-TS (pET17b*Pv*DHFR-TS) were included as negative and positive control, respectively.

2.4. Preparation of PkDHFR recombinant protein

BL21(DE3) *E. coli* was transformed using pET17b*Pk*DHFR-TS. Transformants were picked and cultured in LB broth supplemented with 100 μg/mL ampicillin at 37 °C until cell density reached to OD_{600} of 1, when IPTG was added at a final concentration of 0.4 mM and the growth temperature was reduced to 16 °C. Cells were further incubated for overnight (~15 h). Cell culture was pelleted by centrifugation at 4 °C 8,000 × *g* for 15 min and resuspended in buffer I (20 mM potassium phosphate pH 7, 0.1 mM EDTA, 10 mM DTT, 50 mM KCl, and 20% (v/v) glycerol). Bacterial lysate was prepared by applying cell suspension through French Press. Bacteria cell lysate was centrifuged at 15,000 × *g* 90 min to separate soluble protein fraction from insoluble fraction. The protein was purified using Methotrexate-Sepharose (1.25 × 2 cm) pre-equilibrated with buffer I. Unbound proteins were eliminated by washing the column with buffer II (20 mM potassium phosphate pH 7.0, 0.1 mM EDTA 0.5 M KCl, 30 mM β-mercaptoethanol, 15% (v/v) glycerol) and *Pk*DHFR-TS was eluted using buffer III (50 mM TES pH 8.2, 0.1 mM EDTA, 10 mM DTT, 50 mM KCl, 20% (v/v) glycerol, and 4 mM H_2folate). The protein was further purified by Q-Sepharose column (1.25 × 8 cm) pre-equilibrated with buffer III but without H_2folate. The protein was eluted with a linear gradient from 50 to 500 mM KCl in buffer III (500 mL total volume). Purified *Pk*DHFR-TS was kept in buffer I at −80 °C until used.

The native molecular mass of *Pk*DHFR-TS was determined using Superdex 200 HR 10/300 column (GE Healthcare Life Sciences, Pittsburg, PA, USA) pre-equilibrated with buffer IV (50 mM potassium phosphate pH 7, 150 mM KCl and 5% glycerol). Reference proteins were thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), and ribonuclease A (13.7 kDa).

2.5. Enzyme activity assays and kinetic studies

DHFR activity of *Pk*DHFR-TS was performed in 1 mL reaction containing 50 mM potassium phosphate pH 6, 10 mM β-mercaptoethanol, 1 mM EDTA, 1 mg/mL BSA, 50 μM H_2folate, 100 μM NADPH, and 0.007 μM enzyme. The reaction progression was monitored for reduction of absorbance 340 nm and the DHFR activity was calculated based on ε_{340} of 12,300 M^{-1} cm^{-1} [24]. K_m values for H_2folate and NADPH of *Pk*DHFR were determined by performing the reaction as described above but in the presence of 100 μM NADPH and 0.39–50 μM H_2folate, and 50 μM H_2folate and 0.78–100 μM NADPH, respectively.

For TS activity, 1 mL reaction contained MTA-TS polybuffer pH 8.5 (50 mM acetic acid, 50 mM MES, 100 mM Tris-Cl, 10 mM β-mercaptoethanol, 25 mM $MgCl_2$, 6.5 mM HCOH, 1 mM EDTA and 75 mM β-mercaptoethanol), 100 μM (6*R*)-CH_2H_4folate (Merck Eprova AG, Switzerland), 125 μM dUMP, and 0.08 μM enzyme. Increasing of absorbance 340 nm was monitored and the TS activity was calculated based on ε_{340} of 6400 M^{-1} cm^{-1} [25]. The K_m values for (6*R*)-CH_2H_4folate and dUMP of TS enzyme were determined at a constant concentration of dUMP (125 μM) and varying the concentrations of (6*R*)-CH_2H_4folate (6.25–200 μM) and 100 μM (6*R*)-CH_2H_4folate, 2–125 μM dUMP, respectively.