SEQUENCE ANALYSIS OF THE GENE ENCODING 1-DEOXY-D-XYLULOSE 5-PHOSPHATE (DOXP) REDUCTOISOMERASE IN MULTIDRUG RESISTANT ISOLATES OF *PLASMODIUM FALCIPARUM* COLLECTED FROM PATIENTS ALONG THAI-MYANMAR BORDER AREAS

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Abstract

The isoprenoid biosynthesis pathway in *Plasmodium falciparum* has shown to be the effective drug target of malaria treatment. The new antimalarial drug, fosmidomycin and its derivative FR900098 acts through inhibition of the 1-deoxy-D-xylulose 5-phosphate (DOXP) reductoisomerase which is the second enzyme in the reaction cascade of the DOXP pathway. In recent clinical trials performed in Thailand, fosmidomycin proved to be efficient in the treatment of patients suffering from acuted, uncomplicated *P. falciparum* malaria. However, the sequence diversity of the gene encoding DOXP reductoisomerase in malaria parasites has not been reported. Here we show for the first time that the DOXP reductoisomerase gene is highly conservation among multidrug resistant *P. faciparum* isolates collected from patients along Thai-Myanmar border areas; Maesod district, Tak province, Saiyok district, Kanchanaburi province and Maungranong district, Ranong provinces of Thailand that mean the DOXP reductoisomerase are also conserved among *P. falciparum* in different geographical areas in Thailand. Therefore, inhibiting the growth of multidrug resistant *P. faciparum* isolates by DOXP reductoisomerase inhibitors will be consistency. The finding suggested that the isoprenoid synthesis pathway is attractive to identify for inhibitors for the future drug target for malaria chemotherapy.

Keywords: 1-deoxy-D-xylulose 5-phosphate (DOXP) reductoisomerase, *Plasmodium falciparum*, Fosmidomycin

Introduction

Malaria has been a major problem of public health in endemic area for several decades mostly in the world's poorest countries. Malaria incidence remains high with more than 3000 African children dying daily[1]. The emergence of drug resistance is a major obstacle to the effective treatment and control of the disease. *Plasmodium falciparum* is the most virulent and fatal of the human malaria parasites and has been developed resistance to nearly all available antimalarial currently in used[2]. Therefore,

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new antimalarial drugs are urgently needed.

The isoprenoid biosynthesis pathway in *P. falciparum* is one of the promising pathways for development of new antimalarial drug. Isoprenoids play important roles in all living organisms, they act as steroid hormones in mammals, carotenoids in plants and ubiquinone or menaquinone in bacteria[3]. A new pathway to the isoprenoid precursor, isopentenyl diphosphate (IPP) was discovered in early 1990s and so call a non-mevalonate pathway[4]. In contrast to the well-known mevalonate pathway, it includes 1-deoxy-D-xylulose-5-phosphate (DOXP) as a key metabolite. Several organism including *P. falciparum* rely on this new DOXP pathway. Therefore, all enzymes in this pathway are attractive targets for development of new antimalarials drug.

The gene encoding enzyme for the DOXP reductoisomerase in *P. falciparum* were identified and characterized[5]. Interestingly, high degree of similarity have been revealed between DOXP gene of *P. falciparum* and known bacterial suggesting that the functional of DOXP reductoisomerase in *P. falciparum* is crucial for this parasite. The new antimalarial drug, fosmidomycin and its derivative FR900098 acts through the inhibition of the 1-deoxy-D-xylulose-5-phosphate reductoisomerase which is the second enzyme in the reaction cascade of the DOXP pathway. In recent report, fosmidomycin and its derivative proved to be efficient in the treatment of patients suffering from acuted, uncomplicated *P. falciparum* malaria[6-11]. However, the sequence diversity of the gene encoding DOXP reductoisomerase in malaria parasites has not been reported.

In this study the polymorphism of the DOXP reductoisomerase gene among multidrug resistant *P. faciparum* isolates collected from patients along Thai-Myanmar border areas; Tak, Kanchanaburi and Ranong provinces of Thailand were analyzed and compared.

Materials and Methods

Samples collection from study sites

Finger prick blood samples were obtained from patients attending at three malaria clinics, Saiyok district, Kanchanaburi province, Maesod district, Tak province and Maung Ranong district, Ranong province. Fifty microliters of blood per patient was kept in transporting medium and transported to perform *in vitro* continuous culture in the laboratory located at Bangkok.

Parasite culture

One *P. falciparum* clone (T9/94RC17) and seven isolates from field were propagated in continuous culture using the method described by Trager and Jensen[12]. The culture was harvested at a parasite of about 10 %, wash once with PBS, the parasite was then be ready for DNA extraction.

In vitro drug susceptibility test

The well growing parasites from continuous culture were tested for drug susceptibility[13] with mefloquine, pyrimethamine, chloroquine and quinine. Morphology of parasites and minimum inhibition concentration value of each drug was observed.

Genomic DNA extraction

DNA was extracted from asynchronous parasite cultures by a modification of a previously described method[14]. After lysis of infected erythrocytes in 0.05% saponin in PBS for 10 min at room temperature, the pelletted parasites were washed three time with PBS and then lysed with 0.5 mg/ml protenase K in lysis buffer, 40 mM Tris-HCl pH 8.0, 80 mM EDTA pH 8.0 and 2% SDS for 1 to 2 hours. After extraction with phenol and chloroform, the

DNA was precipitate and immediately dissolved in TE buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA). The DNA samples were then determined the concentration by spectrophotometer.

In vitro amplification

The sequences of reductoisomerase gene of eights *P. falciparum* clone and isolates were amplified by PCR[15] using two set of overlapping oligonucleotides. The PCR mixture consisted of 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM deoxynucleotide triphosphates, 0.2 µM primer and 1.0 U of DNA polymerase (Promega, Madison, USA.) to a totol volume of 50 µl. The PCR was performed by using a GeneAmp® PCR System 9700. The temperature cycle consisted of denaturation (95 °C, 1 min), annealing (57 °C, 1 min) and extension (72 °C, 2 min) for 35 cycles. DNA from malaria parasite clone T9/94RC17 and purified water were used as positive and negative control, respectively. The amplified product was analysed by agarose gel electrophoresis.

Agarose gel electrophoresis

The amplified product (5 µl) was separated by 2% agarose gel electrophoresis and the present or absent of 1,467 base pair band was determine after ethidium bromide staining. The amplified product was purified by PCR Purification Kit (QIAquick®, Qiagen, Germany) prior to perform DNA sequencing.

DNA sequence analysis

DNA sequence was determined by the dideoxynucleotide chain-termination method[16] with an automated sequencer and the protocol of the supplier (ABI, Applied Biosystem). A homology search was performed by DNASIS programme.

Results and Discussion

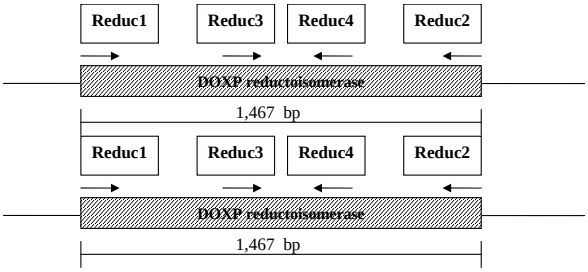
Seven *P. falciparum* infected blood samples were collected from malaria patients attended malaria clinics in different endemic areas along Thai-Myanmar border. Four isolates (K56, K60, K63, K66) were taken from patients in Saiyok district, Kanchanaburi province. RN6 and RN7 were obtained from patients in malaria clinic in Ranong province and T139 was from Mae Sod district, Tak province. T9/94RC17, a pure clone *P. falciparum,* well adapted in *in vitro* cultivation were used as a control. All parasites isolates and clone were propagated *in vitro* culture and antimalarial drug susceptibility were tested as a results were shown in table 1. The parasites DNA were extracted by modification of phenol/chloroform method. The two set of overlapping oligonucleotide primers were designed and synthesized, the sequence and the location of the primers on the DOXP reductoisomerase gene were shown in figure 1. The DOXP reductoisomerase gene was then amplified by using a GeneAmp® PCR System 9700 and the PCR condition was also optimized, the PCR product size 1,467 base pairs was analysed by agarose gel electrophoresis as shown in figure 2. The PCR product was purified by PCR Purification Kit and the whole sequences of DOXP reductoisomerase gene was analysed by the dideoxynucleotide chain-termination method with an automated sequencer and the protocol of the supplier. A homology search was performed by DNASIS programme.

The results from drug susceptibility test revealed that all seven parasite isolates collected from the fields were multidrug resistant as they are all resisted to chloroquine, mefloquine, pyrimethamine and quinine. The comparison of nucleotide sequences of DOXP reductoisomerase gene from the studied isolates to both T9/94RC17 and HB3 (sequences obtained from database at the Institute for Genomic Research, TIGR) indicating that this gene is highly conserved with one hundred percent similarity at

nucleotide and protein level in all seven isolates and one clone of *P. falciparum*, the nucleotide sequences was shown in figure 3. From this study we could determine the potential of the enzyme DOXP reductoisomerase of *P. falciparum* to be an attractive alternative antimalarial drug target in the future.

Table 1 Drug susceptibility of seven isolates and one clone of *P. falciparum* to four antimalarial drugs, chloroquine, pyrimethamine, mefloquine and quinine. Concentrations causing minimum inhibition were determined *in vitro*.

P. falciparum Isolates and clone	Place of origin	MIC (Mol/liter)			
		Chloroquine	Pyrimethamine	Mefloquine	Quinine
T9/94RC17	Tak	5×10^{-7}	5×10^{-8}	2×10^{-7}	5×10^{-7}
T139	Tak	5×10^{-7}	10^{-4}	2×10^{-7}	3×10^{-7}
K56	Kanchanaburi	5×10^{-7}	2×10^{-4}	2×10^{-7}	3×10^{-7}
K60	Kanchanaburi	5×10^{-7}	2×10^{-4}	2×10^{-7}	3×10^{-7}
K63	Kanchanaburi	5×10^{-7}	10^{-4}	2×10^{-7}	3×10^{-7}
K66	Kanchanaburi	10^{-4}	10^{-4}	2×10^{-7}	5×10^{-7}
RN6	Ranong	5×10^{-7}	10^{-4}	2×10^{-7}	3×10^{-7}
RN7	Ranong	5×10^{-7}	10^{-4}	2×10^{-7}	3×10^{-7}



Forward primers
Reduc15' ATG AAG AAA TAT ATT TAT ATA TAT TTT TTC 3'
Reduc35' TCC ATT GTC TCT GCT GGT TTC 3'

Reverse primers
Reduc25' CTA TGA AGA AGT ATG TTT GTT GTA TAT ATC 3'
Reduc45' TTT GAA ATG GAC CTC CAG ATG 3'

Figure 1 Two set of oligonucleotides, forward primers (Reduc 1 and Reduc 3) and reverse primers (Reduc 2 and Reduc 4) were design and synthesized.

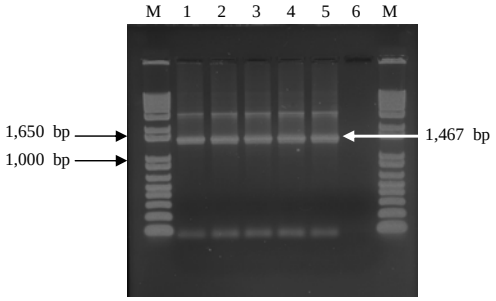


Figure 2 Agarose gel electrophoresis of DOXP reductoisomerase gene product amplified by PCR: Lane M = 1 Kb Ladder marker, Lane 1 = T139, Lane 2 = RN6, Lane 3 = RN7, Lane 4 = K56, Lane 5 = K66, Lane 6 = Negative control

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   1  atgaagaaat atatttatat atatttttc ttcatcacaa taactattaa tgatttagta   60
  61  ataaataata catcaaaatg tgtttccatt gaaagaagaa aaaataacgc atatataaat  120
 121  tatggtatag gatataatgg accagataat aaaataacaa agagtagaag atgtaaaaga  180
 181  ataaagttat gcaaaaagga tttaatagat attggtgcaa taaagaaacc aattaatgta  240
 241  gcaatttttg gaagtactgg tagtataggt acgaatgctt taaatataat aagggagtgt  300
 301  aataaaattg aaaatgtttt taatgttaaa gcattgtatg tgaataagag tgtgaatgaa  360
 361  ttatatgaac aagctagaga atttttacca gaatatttgt gtatacatga taaaagtgta  420
 421  tatgaagaat taaaagaact ggtaaaaaat ataaaagatt ataaacctat aatattgtgt  480
 481  ggtgatgaag ggatgaaaga aatatgtagt agtaatagta tagataaaat agttattggt  540
 541  attgattctt ttcaaggatt atattctact atgtatgcaa ttatgaataa taaaatagtt  600
 601  gcgttagcta ataaagaatc cattgtctct gctggtttct ttttaaagaa attattaaat  660
 661  attcataaaa atgcaaagat aatacctgtt gattcagaac atagtgctat atttcaatgt  720
 721  ttagataata ataaggtatt aaaaacaaaa tgtttacaag acaatttttc taaaattaac  780
 781  aatataaata aaatattttt atgttcatct ggaggtccat ttcaaaattt aactatggac  840
 841  gaattaaaaa atgtaacatc agaaaatgct ttaaagcatc ctaaatggaa aatgggtaag  900
 901  aaaataacta tagattctgc aactatgatg aataaaggtt tagaggttat agaaacccat  960
 961  tttttatttg atgtagatta taatgatata gaagttatag tacataaaga atgcattata 1020
1021  cattcttgtg ttgaatttat agacaaatca gtaataagtc aaatgtatta tccagatatg 1080
1081  caaataccca tattatattc tttaacatgg cctgatagaa taaaaacaaa tttaaaacct 1140
1141  ttagatttgg ctcaggtttc aactcttaca tttcataaac cttctttaga acatttcccg 1200
1201  tgtattaaat tagcttatca agcaggtata aaaggaaact tttatccaac tgtactaaat 1260
1261  gcgtcaaatg aaatagctaa caacttattt ttgaataata aaattaaata ttttgatatt 1320
1321  tcctctataa tatcgcaagt tcttgaatct ttcaattctc aaaaggtttc ggaaaatagt 1380
1381  gaagatttaa tgaagcaaat tctacaaata cattcttggg ccaaagataa agctaccgat 1440
1441  atatacaaca aacataattc ttcatag                                     1467
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Figure 3 Nucleotide sequence of DOXP reductoisomerase gene of one clone, T9/94RC17 and seven isolates of *P. falciparum*, T139, K56, K60, K63, K66, RN6 and RN7 that show 100 percent identity.

การวิเคราะห์ลำดับเบสของยีนรีดักโตไอโซเมอเรสในเชื้อพลาสโมเดียมฟัลซิพารัมที่ดื้อยาต้านมาลาเรียจากคนไข้ในแถบชายแดนไทย-พม่า

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บทคัดย่อ

ในการศึกษากระบวนการสร้างสารไอโซพีนอยของเชื้อพลาสโมเดียม ฟัลซิพารัม ทำให้ทราบว่าการยับยั้งขั้นตอนใดขั้นตอนหนึ่งของกระบวนการนี้มีศักยภาพที่จะนำมาใช้เป็นเป้าของยาต้านมาลาเรียได้เป็นอย่างดี และได้มีการผลิตยาชนิดใหม่ชื่อ ฟอสมิโดมัยซิน และอนุพันธุ์ของยาชนิดนี้คือ FR900098 ซึ่งเป็นยาที่ยับยั้งการสร้างเอ็นไซม์ดีออกซีเซลลูโรสฟอสเฟตรีดักโตไอโซเมอเรส ซึ่งเป็นเอ็นไซม์ในลำดับที่สองของกระบวนการสร้างไอโซพีนอยของเชื้อมาลาเรีย จากผลการทดลองทางคลินิกในผู้ป่วยมาลาเรียชนิดไม่รุนแรงในประเทศไทยพบว่ายานี้รักษาได้ผลดี อย่างไรก็ตามยังไม่เคยมีการศึกษาถึงความหลากหลายของยีนที่สร้างเอ็นไซม์ดีออกซีเซลลูโรสฟอสเฟตรีดักโตไอโซเมอเรสของเชื้อมาลาเรียในประเทศไทย ทางผู้วิจัยจึงได้ทำการเก็บตัวอย่างเชื้อมาลาเรียจากผู้ป่วยที่เข้ารับการรักษาที่มาลาเรียคลินิก 3 แห่งคือ มาลาเรียคลินิก อำเภอแม่สอด จังหวัดตาก อำเภอไทรโยค จังหวัดกาญจนบุรี และ อำเภอเมืองระนอง จังหวัดระนอง ซึ่งเป็นบริเวณเขตชายแดนไทย-พม่าซึ่งเชื้อมาลาเรียชนิดฟัลซิพารัมมีการดื้อต่อยาต้านมาลาเรียแบบ multi-drug resistance หมายถึงเชื้อดื้อต่อยาตั้งแต่3 ชนิดขึ้นไป จากผลการศึกษาลำดับเบสทั้งหมดของยีนดีออกซีเซลลูโรสฟอสเฟตรีดักโตไอโซเมอเรสในเชื้อมาลาเรียชนิดฟัลซิพารัมจำนวน 1 สายพันธุ์บริสุทธิ์และ 7 ไอโซเลตพบว่ายีนนี้มีการอนุรักษ์สูง ไม่มีการกลายพันธุ์เกิดขึ้นในยีนนี้ นั่นย่อมหมายความว่าเอ็นไซม์ที่ยีนนี้สังเคราะห์จะมีการอนุรักษ์สูงเช่นกันดังนั้นการขัดขวางการทำงานของเอ็นไซม์ชนิดนี้จึงสามารถยับยั้งการเจริญเติบโตของเชื้อพลาสโมเดียม ฟัลซิพารัมที่ดื้อต่อยาต้านมาลาเรียได้อย่างแน่นอน กระบวนการสร้างไอโซพีนอยของเชื้อมาลาเรียจึงน่าจะใช้เป็นเป้าหมายในการผลิตยาต้านมาลาเรียได้ดีในอนาคต

คำสำคัญ: ดีออกซีเซลลูโรสฟอสเฟตรีดักโตไอโซเมอเรส พลาสโมเดียม ฟัลซิพารัม ฟอสมิโดมัยซิน

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