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トラベラーズワクチンの現状と課題


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4．ワクチン接種・治療の実際
1）マラリア

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はじめに

マラリアは有史以前から人類の健康を損ない，その生命をも奪ってきた疾患である．しかも，いまなお熱帯，亜熱帯各地を猖獗してやまず[1)]，年間３億人以上が罹患し，幼児を中心に200万人以上が犠牲になっており，非流行地では熱帯地からの輸入マラリアの増加に悩まされ，その対策に有効なマラリアワクチンの開発が望まれている．しかし，叡智を尽くした研究が推進されて久しいが，未だ実用的なマラリアワクチンは開発されておらず，その治療は化学療法に依存せざるを得ないのが現状である．

マラリアワクチン開発の曙光

一概に感染症といっても，その病因はウイルスから多細胞の真核生物の蠕虫まで多種多様である．このうち，天然痘や黄熱などは重篤な疾患ではあるが，その獲得免疫は長期間存続し，宿主を次の感染ないしは発症抑止に導くことから，弱毒生ワクチンまたは不活化ワクチンの接種による防御が応用されている．さらに，他の多くのウイルス性疾患，細菌性疾患には，その種別により経口・非経口生ワクチン，不活化ワクチン，生菌，生・死菌ワクチン，リコンビナント，蛋白多糖体，トキソイドなど，多種多様なワクチンが開発され，既に実用化されている．しかし，真核生物であるマラリア原虫の場合は，ウイルスや細菌よりも高度に寄生適応しており，宿主の免疫応答を回避するエスケープ機構を有することが，ワクチン開発のネックになっている．

一方，これまでのマラリアワクチン研究の成果をみると，大阪大学微生物病研究所（阪大微研）の堀井俊宏教授らが人工遺伝子を用いて熱帯熱マラリア原虫のSERA蛋白質の*N*-末端領域を大腸菌に発現させ，それがマラリア原虫に対する標的抗原の１つであることを明らかにしたことが注目され[2)]，ワクチン開発に向けての研究の今後の発展が期待されている．いずれにしても，マラリア原虫の遺伝子解析により，原虫増殖を抑制する抗体の標的抗原の遺伝子多型や多重族遺伝子群形成による抗原性の変化のほか，マラリア患者の血清中には多数の原虫抗原に対する多量の抗体が産生されるが，その多くは防御に働かないこともワクチン開発を困難にしている．しかし，マラリアワクチン開発は，その有効性と持続期間はもとより，安全性と他の予防接種に干渉しないことが重要な要件である．

マラリア治療の原理と問題点

人類の宿願であったマラリアワクチン開発の曙光がみえてきたとはいえ，マラリアの感染・発症を抑制するワクチンは現段階では未開発の状態にあり，マラリア制圧の重要な一翼を担っているのは治療であり，化学療法が基本である．なかでも，この化学療法では，全種マラリアの赤血球内無性原虫をターゲットにした

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急性期の発熱抑止療法と，三日熱と卵形マラリアでは発熱抑止療法に引き続き，肝細胞内発育環の休眠原虫(ヒプノゾイト)を殺滅して以降の再発阻止を図る根治療法が重要である．

マラリアの発熱抑止療法に適用される化学療法薬を殺シゾント薬と呼び，最初に使用されたのはキナ樹皮で，17世紀中葉に原産地のペルーからヨーロッパに導入され，300年以上もマラリア治療に使用されてきた．このキニーネに代わる合成薬として1934年にドイツで初めて開発されたクロロキンは，抗マラリア活性が高く，キニーネよりも毒性が低く，小児や妊婦にも投与できるため，第二次世界大戦後に広範に使用されるようになり，1950年代には画期的な抗マラリア薬として不動の地位を確立していた．しかし，1957年にはタイ，1960年にはコロンビアにおいて，4種マラリア原虫の中で最も悪性の熱帯熱マラリア原虫のクロロキン耐性株が出現して以降，急速に熱帯各地にも出現または拡散し[3]，今日ではクロロキンに感受性を示すのは中近東と中米の一部に分布する熱帯熱マラリア原虫に限られ，熱帯熱マラリア治療の隘路になっている．そこで，この薬剤耐性マラリアの克服のため，1970年代以降に新規開発されたスルファドキシン・ピリメタミン合剤，メフロキン，ハロファントリン，アルテミシニンおよびその誘導体(アーテメター，アーテスネートなど)，アトバコン・プログアニル合剤，アーテメター・ルメファントリン合剤などのほか[4]，1970年代にその効果が再評価された古典的な硫酸キニーネとテトラサイクリン系抗生物質との併用などによる治療が現在は行われている[5]．

しかし，タイと周辺諸国との国境地帯，アマゾン川流域，東アフリカ諸国の特定地域では，既にクロロキンと新規薬剤との交差耐性株も出現しており，その治療は一層困難になっている．また，これらの多剤耐性マラリアにはキニーネの感受性が高いとされてきた．しかし，最近はその感受性低下も東南アジアやブラジルなどから報告されているが，未だ，1990年代に開発されたアトバコン・プログアニル合剤やアーテメター・ルメファントリン合剤のほか，中国最古の医書である黄帝内経に記載され，古くから民間療法に用いられてきたヨモギ科の植物から1970年代に有効成分が確定された青蒿素(quinghaosu＝artemisinine)とその誘導体の治療効果が高いとされている．なお，新規薬剤のハロファントリンは，治療効果は高いが，心電図上のQT延長から心室性不整脈の出現による死亡例の発生などの有害反応があり，次第に使用されなくなっている．

合併症を併発した熱帯熱マラリアの重症例には，キニーネ塩基8.3 mg/kgを200～500 mLの5％ブドウ糖液や生理食塩水に溶解，4時間かけて点滴静注し，必要に応じて8～12時間ごとに繰り返す非経口療法の救命率が高く，軽快したらキニーネの最終投与12時間後にメフロキンなどを経口投与する．さらに，最近は即効性の高いアルテミシン誘導体のアーテメターの筋注，アーテスネートの静注もしくは座薬も効果的であるとする報告が多い．なお，最近の新規抗マラリア薬の中には，従来の薬剤と化学構造，作用機序や薬物動態，特に消失半減期が著しく異なる合剤が少なくないが，これは治療効果の増強を図ると同時に，耐性株出現の阻止，あるいはできるだけ遅延させることを狙ったものである．

三日熱，四日熱，卵形マラリアに対する発熱抑止療法の選択薬はクロロキンである．しかし，1989年にパプア・ニューギニアから三日熱マラリア原虫のクロロキン耐性株の出現が報告され，1990年代にはインドネシア，ミャンマー，バヌアツなどでも耐性株の出現が確認され，クロロキンによる治療が奏効しないときは，メフロキンによる治療に変更する．

さらに，肝細胞発育環のヒプノゾイトを有する三日熱と卵形マラリアでは，クロロキンなどによる発熱抑止療法後にプリマキンでそれを殺滅しないと，その後の再発阻止が困難になる．しかし，パプア・ニューギニアには1950年代からプリマキン低感受性のChesson株の分布が知られており，最近は東南アジア各地からも低感受性株の存在が報告されている．そのため，プリマキンの標準投与(15 mg塩基/日，14日間)を1カ月の間隔を置いてさらに1クール繰り返したり，1日当たりの投与量を22.5 mgに増量する方法などが案出されている[6]．しかし，増量によりG6PD欠損症患者以外にも溶血を誘導する危険があり，より安全性の高い用法・用量の検討のほか，効果的で安全性の高い新規薬剤の開発が望まれている．いずれにしても，抗マラリア薬は，原虫の種類とその発育環に特異的に作用する特性を念頭に置き，特に熱帯熱マラリアの場合は，患者の病態，合併症発現の有無，推定感染地における薬剤耐性株の分布状況を考慮して，最も適切な薬剤の選択による迅速な治療を開始するか否かが，患者の予後に直接関与することを肝に銘ずるべきである．

表1　国内で入手可能な抗マラリア薬とその用法

商品名・剤型	一般名・含量	用法・用量(成人量)
Ⅰ．合併症のない薬剤耐性熱帯熱マラリアに対する発熱抑止療法		
ファンシダール，錠	スルファドキシン500 mg/ ピリメタミン25 mg	3錠単回服用
メファキン，錠	塩酸メフロキン275 mg	4錠単回服用，または6～8時間間隔で2分服(体重45 kg以下では3錠に減量)
硫酸キニーネ，末	硫酸キニーネ	1.5～1.8 gを分3，7日間服用 テトラサイクリン系薬剤1 g，分4，7日間併用
マラロン*，錠	アトバコン250 mg/ 塩酸プログアニル100 mg	1日1回4錠，3日間，食事または乳製品とともに服用
リアメット*，錠	アーテメター20 mg/ ルメファントリン100 mg	初回，8，24，36，60時間後に各4錠服用
プラスモトリム*，錠・座薬	アーテスネート50 mg，200 mg	初日200 mg 2錠，分2 第2～5日1内服または直腸内挿入
Ⅱ．重症熱帯熱マラリアに対する発熱抑止療法		
キニマックス*，注	2 mL中にグルコン酸キニーネ：キニーネ塩基240 mg，他のアルカロイドを含む全塩基250 mg	キニーネ塩基8.3 mg/kgを200～500 mLの5％ブドウ糖液や生理食塩水に希釈し，4時間かけて点滴静注．必要に応じ8～12時間ごとに繰り返し，軽快したら経口薬に切り替える． なお，1回量を算出するには，体重÷15.1(単位mL)をアンプルから吸い，5％ブドウ糖液で希釈すればよい．
プラスモトリム*，座薬	アーテスネート500 mg	用法・用量は上記と同じ
Ⅲ．三日熱，四日熱，卵形マラリアに対する発熱抑止療法		
ニバキン*	硫酸クロロキン150 mg塩基	クロロキン塩基を初回600 mg，6，24，48時間後に各300 mg服用
ファンシダール3錠単回服用，硫酸キニーネ1.2～1.5 gの3～5日間内服でもよい．		
Ⅳ．三日熱，卵形マラリアに対する根治療法		
プリマキン*，錠	リン酸プリマキン15 mg塩基	1日1回，15 mg塩基，14日間服用

*：「熱帯病治療薬研究班」から入手可能．

治療の実際と注意点

重要なことは，治療中は患者の病状経過を厳重に監視すると同時に，経時的に血液塗抹ギムザ染色標本の鏡検により赤血球原虫感染率(虫血症)の推移と赤血球内無性原虫の変性像を観察，さらに原虫消失時間(PCT)と発熱消失時間(FCT)を確認することが，治療効果の判定に不可欠である．特に熱帯熱マラリアの場合は，少なくとも1日数回はこの血液検査を行い，化学療法を開始して2～3日以内に虫血症の減少・消失が認められなかったり，増加するときは選択薬に耐性と判断し，薬剤の変更を考慮する．ただし，選択薬により，即効性のものと遅効性のものとがあり，一般にスルファドキシン・ピリメタミン合剤は遅効性，アトバコン・プログアニル合剤はやや遅効性，アルテミシニン誘導体は即効性であるので，選択薬の薬剤特性を理解しておくことも必要である．

表1に国内で入手可能な抗マラリア薬の用法・用量を示したが，薬剤によりその特性が異なる．

1．スルファドキシン・ピリメタミン合剤

東南アジア，アフリカ，南米の一部などに耐性株が存在し，本剤の治療効果は期待できない．本剤過敏症，妊婦，授乳婦，新生児，G6PD欠乏症患者には禁忌．スルホンアミドと薬物相互作用．まれに中毒性表皮壊死，Stevens-Johnson症候群などの重篤な副作用．薬価は1錠484.2円．

2．メフロキン

治療効果は高いが，既に耐性株出現．本剤・キニーネ過敏症，新生児，妊婦，痙攣既往者，精神病患者には禁忌．抗不整脈薬，Ca拮抗薬，β遮断薬などとの併用注意．副作用としてめまい，ふらつき，頭痛，悪夢，まれに中毒性表皮壊死．経口腸チフスワクチン，狂犬病ワクチンに対する干渉．CYP3A阻害薬，CYP3A誘導薬と薬物相互作用．薬価は1錠854.8円．

3．アトバコン・プログアニル合剤

薬剤耐性熱帯熱マラリアを含む全種マラリアに有効．リファンピシンとの併用でアトバコンのAUC(血中濃度－時間曲線下面積)は約50%低下．腹痛，悪心，嘔吐などの副作用があるが，概して軽度．国内未発売．

4．アーテメター・ルメファントリン合剤

薬剤耐性熱帯熱マラリアに有効．腹痛，下痢，頭痛，めまいなどの副作用．グレープフルーツジュースでの服用で吸収低下．国内未発売．

5．アーテスネート

アルテミシニンのコハク酸塩で，多剤耐性マラリアや重症マラリアの治療に速やかに反応．副作用は少なく，小児，妊婦にも使用できる．しかし，消失半減期が短いことから作用時間が短く，再燃率が高いので，メフロキンなどでの追加療法が有効[7]．薬物相互作用としてはCYP3A4の阻害薬．国内未発売．

6．キニーネ

経口投与には硫酸塩，時に塩酸塩，静脈内投与には二塩酸キニーネまたはグルコン酸キニーネを使用．常用量の副作用として可逆性の耳鳴，高音性難聴，視覚障害，頭痛，悪心，大量投与によりキニーネ中毒，低血糖，血圧降下．G6PD欠乏症には溶血を誘導．本剤過敏症，妊婦には禁忌．薬物相互作用としては，強心配糖体，リトナビル，ワルファリンとの併用により，その血漿濃度を高める．薬価は硫酸キニーネ1g 130.9円，塩酸キニーネ1g 152.8円．二塩酸キニーネ，グルコン酸キニーネの注射薬は国内未発売．

7．テトラサイクリン系薬剤

薬剤耐性熱帯熱マラリアに対するキニーネと併用し，キニーネの治療効果増強．単独では使用しない．また，予防内服には塩酸ドキシサイクリンの単独使用．光線過敏症，めまい，胃腸障害，菌交代症などの副作用．過敏症には禁忌，妊婦，8歳以下の小児には投与しない．ドキシサイクリンによる予防内服は12週を超えてはならない．保険適用外．

8．クロロキン

クロロキン感受性熱帯熱を含む全種マラリアの熱発作治療に有効であるが，熱帯熱マラリア原虫の生殖母体，三日熱と卵形マラリア原虫の休眠原虫には無効．比較的安全性の高い薬剤であるが，胃腸障害，羞明，瘙痒感，長期投与により視覚異常，網膜炎，錯乱などの副作用．てんかん，重症筋無力症患者には禁忌．G6PD欠乏症患者では溶血を起こす．薬物相互作用では，金剤またはフェニルブタゾンとの併用により皮膚炎，アミオダロンとの同時投与では心室性不整脈を倍加させ，ジゴキシンやシクロスポリンとの併用により血漿濃度の増加．国内未発売．

9．プリマキン

三日熱と卵形マラリアの再発阻止を図る根治療法薬であるが，熱帯熱マラリア原虫の生殖母体の駆除にも有効．時に腹痛，腹部不快感を起こす．本剤により急性溶血を誘導するG6PD欠乏症患者と妊婦には禁忌．国内未発売．

重症マラリアに対する支持療法

熱帯熱マラリアは，臨床経過中に高度虫血症，脳症，急性腎不全，ARDS/肺水腫，代謝性アシドーシス，出血傾向，循環不全，重度貧血などの重篤な合併症を発現する危険性が高い．WHOは重症マラリアの判定基準とその治療のガイドラインを策定し，随時改訂している[8]．

この重症マラリアには，上述の特異療法だけでなく，病態に応じた支持療法の強化が患者救命に不可欠である．

国内未承認抗マラリア薬の入手法

現在，国内に流通している抗マラリア薬は，従来からのキニーネ，1987年承認のファンシダール錠(スルファドキシン・ピリメタミン合剤)，2001年承認のメファキン錠(塩酸メフロキン)のみである．そこで，本文中に述べた抗マラリア薬が患者治療に必要な場合は，厚生労働科学研究費創薬等ヒューマンサイエンス総合研究事業「熱帯病治療薬研究班(略称)」(http://ims.u-tokyo.ac.jp/didai/orphan/index.html)にアクセスすれば無償供与されることを知っておくとよい．

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Antimalarial Agents: Current Perspective

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Malaria is still the most serious global protozoan disease in the tropical and subtropical regions. Additionally, the risk of malaria infection in persons traveling in the epidemic area is increasing, but is seldom known in detail. Development of malaria vaccine for preventing infection, disease, and death is urgently needed and now some vaccines are carefully in trials.

The main framework for malaria prevention and control is a larger choice of antimalarial drugs. Chemotherapy of malaria is limited by established drug resistance and back of novel targets. The drug resistance becomes a public health problem worldwide.

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Potent Plasmodicidal Activity of a Heat-Induced Reformulation of Deoxycholate-Amphotericin B (Fungizone) against *Plasmodium falciparum*

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The emergence and spread of drug-resistant *Plasmodium falciparum* continue to pose problems in malaria chemotherapy. Therefore, it is necessary to identify new antimalarial drugs and therapeutic strategies. In the present study, the activity of a heat-treated form of amphotericin B (HT-AMB) against *P. falciparum* was evaluated. The efficacy and toxicity of HT-AMB were also compared with those of the standard formulation (AMB). HT-AMB showed significant activity against a chloroquine-resistant strain (strain K-1) and a chloroquine-susceptible strain (strain FCR-3) in vitro. The 50% inhibitory concentrations of HT-AMB were 0.32 ± 0.03 μg/ml for strain K-1 and 0.33 ± 0.03 μg/ml for strain FCR-3. In the presence of 1.0 μg of HT-AMB per ml, only pyknotic parasites were observed after 24 h of incubation of early trophozoites (ring forms). However, when late trophozoites and schizonts were cultured with 1.0 μg of HT-AMB per ml, those forms multiplied to ring forms but the number of infected erythrocytes did not increase. These results indicate that HT-AMB possesses potent antiplasmodial activity and that the drug is more effective against the ring-form stage than against the late trophozoite and schizont stages. HT-AMB was observed to have little cytotoxic effect against a human liver cell line (Chang liver cells). In conclusion, the results suggest that HT-AMB has promising properties and merits further in vivo investigations as a treatment for falciparum malaria.

Malaria infection due to *Plasmodium falciparum* is a major public health problem in many tropical and subtropical areas. Sporozoites, the infective form of the parasite, are transferred to the human host during a bite by female *Anopheles* mosquitoes, invade hepatocytes, and develop into liver schizonts, which contain large numbers of merozoites. The asexual blood-stage cycle of *P. falciparum* commences when the merozoites released from hepatocytes enter the blood circulation and invade red blood cells (RBCs). In this phase of the cycle merozoites initially develop within RBCs as ring forms and then progress to trophozoites and eventually to schizonts, which rupture and release a new wave of merozoites that invade a new batch of RBCs. Chloroquine (CQ), a blood-stage schizonticidal drug, has been the drug of choice for the treatment of falciparum malaria for several decades, but its clinical utility has been greatly reduced in most areas where CQ-resistant malaria is endemic (1, 22). However, CQ remains the most widely used first-line antimalarial drug because it is well tolerated, safe for pregnant women and young children, efficacious against susceptible strains of *P. falciparum* and the other three human malaria species, and inexpensive. At present, the development of new antimalarial drugs and the use of preexisting drugs in combination are the most important approaches to overcoming the problem of drug resistance.

Amphotericin B (AMB) is a heptaene macrolide antibiotic that is active against fungi and yeasts. Fungizone, the commercially available deoxycholate salt form of AMB, is the drug that is the most widely used for the treatment of deep-seated mycotic infections. This drug is also the recommended second-line treatment for visceral leishmaniasis when conventional tetravalent antimony therapy is inappropriate or ineffective (11, 15). Unfortunately, intravenously (i.v.) administered AMB causes acute side effects, which limit its more extensive clinical use.

One approach to decreasing the toxicity of AMB has been to develop new derivatives or formulations with greater aggregation. Some investigators have reported that heat treatment of Fungizone leads to an increase in the size of aggregated AMB. Heating of AMB at 70°C for 20 min (heat-treated AMB [HT-AMB]) induces a superaggregated form that leads to a new equilibrium. This novel formulation has been associated with reduced toxicity in mammalian cells, while its antifungal activity is retained in vitro and in vivo (4, 10). This formulation is inexpensive and can be used to improve the therapeutic index of AMB against candidiasis and cryptococcosis and to encourage the more widespread use of AMB (13).

The aim of this study was to evaluate the antimalarial activity of HT-AMB against blood-stage parasites of *P. falciparum* in vitro. The efficacy and toxicity of HT-AMB were compared with those of the standard AMB formulation.

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MATERIALS AND METHODS

Cultivation of *P. falciparum*. A CQ-resistant *P. falciparum* strain, strain K-1, which was originally from Thailand, and a CQ-susceptible strain, strain FCR-3, which was originally from The Gambia, were grown asynchronously by the

modified method of Trager and Jensen (17). We used RPMI 1640 medium with glutamine supplemented with 10% human type O serum, 25 mM HEPES, 25 μg of gentamicin (Sigma-Aldrich, St. Louis, Mo.) per ml, sodium bicarbonate, and human type O RBCs in disposable sterile dishes and a controlled atmosphere of 5% CO_2–5% O_2–90% N_2 at 37°C.

Antifungal agents. AMB (injectable Fungizone) was purchased from Bristol Pharma Co. (Tokyo, Japan). A stock solution of AMB was reconstituted in sterile water according to the instructions of the manufacturer. HT-AMB was prepared by heating AMB solutions for 20 min in a water bath at 70°C, as described by Petit et al. (13).

Evaluation of in vitro plasmodicidal effect of HT-AMB. The following procedures were used to evaluate the antimalarial activities of HT-AMB and AMB. Asynchronously cultivated malaria parasites were used. RPMI 1640 medium was supplemented with 0 (control), 0.5, 1.0, 5.0, or 10.0 μg of HT-AMB or AMB per ml. The antifungal drug-supplemented medium was changed every 24 h. Five hundred microliters of a parasitized RBC (pRBC) suspension was placed in each well of a 24-well flat-bottom culture plate (Sumiron; Sumitomo Bakelite Co., Ltd., Tokyo, Japan) with a hematocrit of 5% and an initial parasitemia level of 0.1%. Thin-smear specimens stained with Giemsa solution were made every 24 h, and the level of parasitemia was determined by counting the number of parasites per 10,000 RBCs.

Determination of HT-AMB and AMB IC_{50}s. In vitro drug susceptibility tests were performed as described previously (16). Briefly, synchronous pRBCs showing a parasitemia level of 1% were placed in 24-well culture plates. Synchronization was achieved by treating the pRBCs with 5% D-sorbitol for 30 min at room temperature. Twenty microliters of drug-supplemented medium was added to each well to give a series of doubling dilutions from 0.10 to 100.00 μg/ml. After 24 h of incubation in an atmosphere of 5% CO_2–5% O_2–90% N_2 at 37°C, the control wells were checked for parasite growth. When the schizonts in the control wells were fully grown, the culture plates were removed from the incubator. Thin-smear specimens were prepared and stained with Giemsa solution. The numbers of RBCs in the control smears were counted under a microscope until 50 schizonts were encountered. The effects of the drugs on parasite growth were evaluated by the observation of decreased numbers of schizonts per equal numbers of RBCs counted previously in the control cultures. The growth inhibition effect (in percent) was calculated as follows: (test well schizont count/control well schizont count) × 100. The 50% inhibitory concentrations (IC_{50}s) of AMB and HT-AMB were calculated by the probit method.

Effect of HT-AMB on a hepatic cell line. Cells of the Chang human liver cell line were a kind gift from Takeaki Nagamine, Gunma University School of Health Sciences (Gunma, Japan). The cells were grown continuously in complete Dulbecco modified Eagle's medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum in a 5% CO_2 atmosphere at 37°C. Prior to exposure to the drugs, the cells were seeded at 10^5 cells/ml in 96-well culture plates and incubated for 72 h in 0.2 ml of Dulbecco modified Eagle medium supplemented with AMB or HT-AMB. Cell death was evaluated by a lactate dehydrogenase release assay (CytoTox 96 assay kit; Promega Corp., Madison, Wis.), according to the protocol recommended by the manufacturer (5). All of the test compounds were assayed at each concentration in triplicate.

Detection of hemolysis caused by treatment with HT-AMB. The level of hemolysis was determined by measuring the amount of hemoglobin that eluted into the medium by the sodium lauryl sulfate method (hemoglobin B test; Wako Pharmaceuticals, Osaka, Japan) described previously (16). Briefly, after exposure of pRBCs or RBCs to 1.0 to 100.0 μg of HT-AMB per ml, the samples were centrifuged at 1,000 × *g* for 5 min at 20°C, and the supernatant was collected and analyzed.

Data analysis. The data are presented as the means ± standard errors of the means from at least three sets of independent experiments. Student's *t* test was used for statistical analysis. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

HT-AMB inhibits *P. falciparum* growth in vitro. To confirm the plasmodicidal activities of AMB and HT-AMB, a CQ-resistant *P. falciparum* strain (strain K-1) and a CQ-susceptible strain (strain FCR-3) were exposed to medium containing 0.5 to 10.0 μg of AMB or HT-AMB per ml for 72 h, and parasite growth and multiplication were monitored (Fig. 1). AMB at concentrations equal to or greater than 1.0 μg/ml induced marked decreases in the levels of parasitemia. HT-AMB at concentrations equal to or greater than 1.0 μg/ml also induced decreases in the levels of parasitemia, but the decrease was slower than that obtained with AMB. At these concentrations, complete inhibition of parasite multiplication was attained within 72 h of incubation.

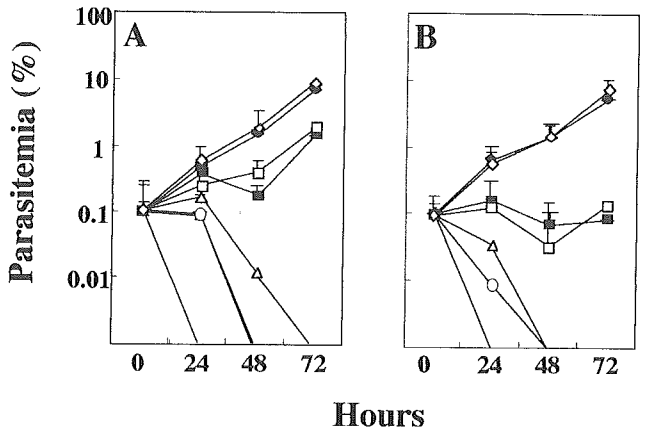

FIG. 1. Effect of HT-AMB on *P. falciparum* parasitemia in vitro. The time- and concentration-dependent effects of continuous incubation with AMB (closed symbols) or HT-AMB (open symbols) on levels of parasitemia caused by CQ-resistant strain K-1 (A) and CQ-susceptible strain FCR-3 (B) of *P. falciparum* are shown. All cultures were started with asynchronized parasites. Parasitemia was measured at the beginning of incubation (0 h) and every 24 h thereafter for 72 h. Parasites were incubated in the presence of drug at concentrations of 0 (diamonds), 0.5 (squares), 1.0 (triangles), and 5.0 (circles) μg/ml.

The results of the in vitro drug susceptibility assay were as follows. The IC_{50}s of AMB were 0.95 ± 0.10 μg/ml for K-1 and 0.89 ± 0.26 μg/ml for FCR-3. The IC_{50}s of HT-AMB were 0.32 ± 0.03 μg/ml for K-1 and 0.33 ± 0.03 μg/ml for FCR-3. The IC_{50}s of HT-AMB were threefold lower than those of AMB. There were no significant differences between the IC_{50}s for CQ-resistant strain K-1 and those for CQ-susceptible strain FCR-3 ($P > 0.05$).

HT-AMB alters *P. falciparum* morphology and interferes with parasite development. The effects of HT-AMB on the morphology and development of *P. falciparum* parasites were evaluated with synchronized cultures of the two strains (CQ-resistant strain K-1 and CQ-susceptible strain FCR-3). The effects against both strains were similar, and the parasites grew to mature stages after 24 h of incubation without HT-AMB (Fig. 2A). When 1.0 μg of HT-AMB per ml was added to synchronized ring-form parasites, pyknotic parasites inside and outside of the RBCs were observed after 24 h of incubation (Fig. 2B). When HT-AMB was added to synchronized late-stage trophozoites and schizonts in culture, parasites that had multiplied but that had altered morphologies at the ring stage were observed after 24 h of incubation (data not shown).

Effect of HT-AMB on Chang liver cells. As shown in Fig. 3, AMB at 12.5 μg/ml showed slight cytotoxicity for Chang liver cells ($P < 0.05$), and AMB at concentrations equal to and greater than 25.0 μg/ml showed strong cytotoxicity, whereas HT-AMB at the same concentrations showed no cytotoxicity ($P < 0.01$).

Hemolysis of pRBCs by HT-AMB. To detect hemolysis as an index of cytotoxicity, the concentration of hemoglobin in the

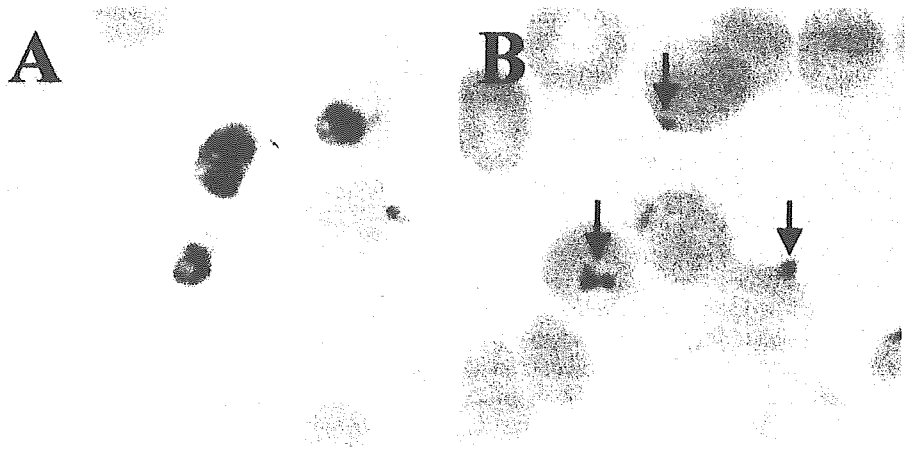

FIG. 2. Morphology of *P. falciparum* K-1 after 24 h of incubation with HT-AMB. Parasites were synchronized at the ring stage. The morphologies of cells in Giemsa-stained thin blood smears from drug-free cultures (A) and cultures incubated with 1.0 μg of HT-AMB per ml (B) for 24 h are shown. Note the parasite pyknotic changes and the prevalence of ring forms in the HT-AMB-treated culture (arrows in panel B). Magnifications, ×1,000.

pRBC or RBC culture medium was determined. In the presence of 1.0 μg of HT-AMB per ml, the concentration of hemoglobin in the pRBC culture medium was significantly higher (0.949 ± 0.192 g/dl) than that in the RBC culture medium (0.138 ± 0.048 g/dl) ($P < 0.05$). Similar results were observed in the case of AMB treatment.

DISCUSSION

Identification of the antimalarial effects of drugs that have been used for other purposes is an attractive approach to overcoming the increasing threat of drug-resistant malaria. AMB is one of the antifungal agents that is the most effective and widely used for the treatment of systemic fungal infections commonly found in immunocompromised patients. However, it can show dose-dependent renal toxicity, which is not predictable by monitoring of the serum drug concentration (19, 20). Several lipid AMB formulations have been developed to decrease this toxicity.

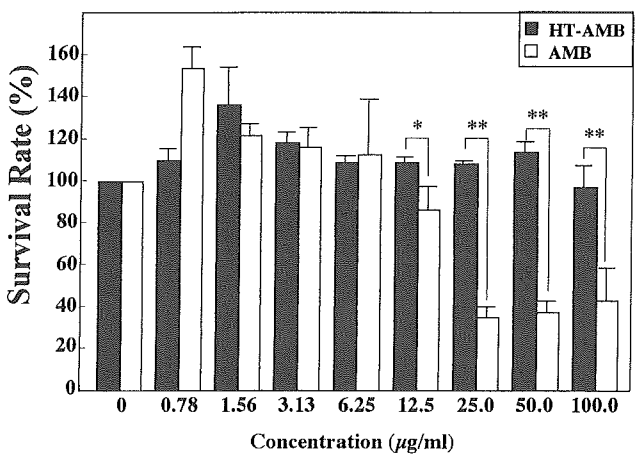

FIG. 3. Effect of HT-AMB on Chang liver cells in vitro. Chang liver cells were cultured for 72 h in the presence of HT-AMB. The viability of the cells was determined with a CytoTox 96 assay kit, which quantitatively measures the amount of lactate dehydrogenase released into the culture medium upon cell death. *, $P < 0.05$; **, $P < 0.01$.

As an inexpensive alternative, simple AMB was treated with moderate heat (70°C for 20 min) to produce a new, self-aggregated state. It has been reported that in vitro HT-AMB exhibits significantly lower levels of toxicity for mammalian renal cells and fewer hemolytic effects against RBCs than the standard formulation and that HT-AMB also shows increased toxicity for fungal cells (4, 10, 13). Our study showed that in vitro HT-AMB has a greater plasmodicidal effect than AMB against both CQ-resistant and CQ-susceptible *P. falciparum* strains. The growth curves of asynchronous parasites showed that AMB has a greater inhibitory effect than HT-AMB, but significant differences were observed only at high concentrations that would not be applicable to treatment for malaria. We confirmed that HT-AMB showed no cytotoxicity for Chang liver cells, as expected, and that HT-AMB showed much less hemolytic activity than AMB. In fact, we conducted in vivo studies of the efficacies of AMB and HT-AMB against malaria parasites using *Plasmodium berghei* NK65 and 20 female ICR/Jcl mice (age, 6 weeks), which consisted of 5 control mice, 5 mice treated with 0.5 mg/kg of body weight i.v., 5 mice treated with 1.0 mg/kg i.v., and 5 mice treated with 2.0 mg/kg i.v. However, no significant difference in parasite growth or the survival rate of the mice was observed (data not shown). AMB and HT-AMB were also not observed to have hemolytic activity. We learned from these experiments that maintenance of effective drug concentrations in the peripheral blood is very important (10), but we were not able to maintain effective drug concentrations by the administration of a single i.v. dose to mice. Further in vivo experiments are still needed before our findings on the effectiveness of AMB and HT-AMB in in vitro studies can be applied to the treatment of both drug-resistant and -susceptible human *P. falciparum* malaria.

Most antimalarial drugs, including CQ, have been reported to show schizonticidal activity in blood. One of the schizonticidal mechanisms of CQ is inhibition of heme polymerization in vitro (2). Another antimalarial drug, quinoline, also inhibits

heme polymerization (2, 7, 8, 12). In this study, pyknotic parasites were observed inside and outside of RBCs when HT-AMB was added to synchronized ring-form cultures at 1.0 μg/ml for 24 h (Fig. 2B). In contrast, when HT-AMB was added to synchronized mature-stage (late trophozoite and schizont) cultures, ring-form parasites that had multiplied successfully invaded and remained inside the RBCs for 12 to 24 h of incubation, indicating that HT-AMB has a greater hemolytic effect against pRBCs than it does against non-pRBCs.

It is generally assumed that the permeabilizing effects of AMB are related to its ability to form transmembrane channels, whereas the lytic effect is due to the peroxidative action of AMB at the membrane level (3, 14, 18). The oxidation of unsaturated fatty acids leads to a change in the membrane, which becomes more sensitive to the osmotic shock induced by channel formation. Autoxidation of AMB in solution as well as AMB-induced peroxidation of unsaturated fatty acids in the RBC membrane is assumed to be triggered by the reactive oxygen species that may be produced by AMB. It has also been reported that increased amounts of reactive oxygen species are generated during malaria infection, leading to RBC membrane damage (6, 9). This may explain the higher levels of plasmodicidal activity and hemolytic activity of HT-AMB against pRBCs, the greater effect of HT-AMB against ring forms than against late trophozoites and schizonts, and the apparently different antimalarial mechanism of HT-AMB compared with those of quinoline antimalarial drugs.

In conclusion, the results of the present study suggest that HT-AMB has promising properties and merits further in vivo investigations for the treatment of falciparum malaria.

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A PILOT FIELD SURVEY ON THE *IN VITRO* DRUG SUSCEPTIBILITY OF *PLASMODIUM FALCIPARUM* IN LAO PDR

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In Southeast Asia, malaria has presented a major public health problem, and the spread of drug-resistant falciparum malaria is making the problem more serious in this region. Thus, evidence-based detection of drug-resistant parasites is important for the accurate evaluation of susceptibility to antimalarial drugs. Lao PDR (Lao People's Democratic Republic) is a developing country in which about 70% of the population lives in malaria endemic areas. Because of the lack of information on the *in vitro* drug susceptibility of parasites in this country, chloroquine (CQ) is still the drug of choice for uncomplicated falciparum malaria [1]. This report is a pilot field survey on the *in vitro* CQ- and mefloquine (MQ)-susceptibility of falciparum malaria using AnaeroPack® gas system in Saravan province, Lao PDR.

Saravan province is located in the southern part of Lao PDR. The survey in this province was conducted from August 8 to 16, 2003. Blood samples were successfully obtained from nine Laotian patients suffering from falciparum malaria. The samples were collected by the staff of the Center of Malariology, Parasitology and Entomology, after explaining the purpose of the study to the patients. The survey was conducted in accordance with the ethical guidelines for epidemiological studies established by the Ministry of Education, Culture, Sports, Science and Technology and Ministry of Health, Labour and Welfare of Japan. The *in vitro* drug susceptibility test was administered using the AnaeroPack® malaria culture system with a thermostat portable incubator as described previously [2, 3]. The AnaeroPack® CO_2 (Mitsubishi Gas Co., Tokyo, Japan) is a foil-packed paper sachet that on exposure to air immediately absorbs atmospheric O_2 and simultaneously generates CO_2 until a condition of 15% O_2 and 5% CO_2 is attained. The microaerophilic atmosphere produced within a sealed jar (AnaeroPack® Kakugata jar, SUGIYAMA-GEN Co., Ltd., Tokyo, Japan) can be maintained for at least 24 hours. The temperature inside the portable thermostat incubator (SUGIYAMA-GEN Co., Ltd.) was adjusted to 37℃. During *P. falciparum* cultivation, the sachet inside the jar was replaced every day when the culture medium was changed. The WHO semi-micro test method was used for evaluation of *in vitro* drug susceptibility [4]. Briefly, blood samples (0.1 ml) were resuspended in RPMI 1640 (GIBCO BRL), pH 7.4, supplemented with 25 mM HEPES, and sodium bicarbonate. To monitor parasite growth, six wells per plate served as controls without antimalarials. When the schizonts were fully grown in the control wells, the culture plate was removed from the incubator. Thin-smear specimens stained with Giemsa solution were made from each well. We defined parasites as schizonts when they had both dark brown pigment and more than three nuclei [5]. The effect of antimalarials on parasite growth was evaluated by the WHO standard evaluation method.

The results of this study are shown in Table 1. When complete schizont inhibition is observed at a CQ amount of

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Table 1: The results for *in vitro* drug susceptibility

No.	Parasitemia (%)	Chloroquine	Mefloquine
A	0.015	Susceptible	Susceptible
B	0.36	Susceptible	Susceptible
C	1.97	Susceptible	Susceptible
D	0.91	**Resistant**	Susceptible
E	0.01	**Resistant**	Susceptible
F	0.13	**Resistant**	Susceptible
G	0.002	**Resistant**	Susceptible
H	0.004	Susceptible	Susceptible
I	0.007	Susceptible	Susceptible

80 nM or less, the parasite is considered susceptible. If schizont formulation is observed at an MQ amount of 640 nM or more, the parasite can be considered resistant. In the present study, four (44%) of the nine isolates were resistant to CQ, while all the isolates were susceptible to MQ. There was no correlation between the parasitemia and CQ-resistance.

The results of this study suggest that CQ-resistant parasites have increased even though CQ is commonly used as the first-line drug for treatment of uncomplicated falciparum malaria in Lao PDR. In neighboring countries such as Thailand and Cambodia, high-grade multi-drug resistant parasites are reported to be spreading and, indeed, *in vivo* CQ-resistant falciparum malaria has already been reported in Lao PDR [6]. Dedicated efforts have to be made to determine the *in vitro* drug susceptibility of *P. falciparum* in Lao PDR as a way to prevent the spread of multi-drug resistant parasites in the near future. This is the first test report on *in vitro* drug resistance in Lao PDR.

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Synthetic Study of an Antigenic Peptide Having a Partial Sequence from *Plasmodium falciparum* Enolase

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The 28-residues peptide having a specific seqeunce from Plasmodium falciparum *enolase* (A^{256}*SEFYNSENKTYDLDFKTPNND*^{277}) *was successfully synthesized by using the combination of stepwise elongation and fragment condensation in solution phase. Fluorescence ELISA data have shown significant reactivity against patients' sera compared with those of healthy volunteers. Thus the peptide has potential applicability as a synthetic antigen such as for diagnnostic usage.*

Keywords: antigen, malaria, peptide, *Plasmodium falciparum* enolase, solution-phase synthesis.

Introduction

Enolase is a key enzyme in the glycolytic pathway and catalyzes the dehydration of 2-phosphoglycerate to 2-phosphopyruvate, which is the ninth reaction in eleven steps pathway from glucose to lactic acid, and the only dehydration reaction in this series [1]. An antigen toward *Plasmodium falciparum* (*P.f.*) enolase has been found in patients' sera [2]. Our previous studies have suggested that a series of partial peptides of *P.f.* enolase have antigenic reactivity against patients' sera. In this study, we have focused on a solution-phase synthesis of an antigenic peptide having a sequence Ala^{256}-Asp^{277} (AD22, Figs 1 and 2) [3], which has been prepared by using solid-phase synthesis in our laboratory. The prepared sequence is shown in Scheme 1.

Results and Discussion

The peptide **1** was synthesized by solution-phase method using both fragment condensation and stepwise coupling procedures as described in Fig. 3. We have chosen *t*-butyloxycarbonyl (Boc) group as α-amino protection. The functional side chains of Asp and Glu were protected with benzyl ester (OBzl). Ser and Thr were protected with benzyl ether (Bzl). Tyr was protected with 2,6-dichlorobenzyl ether(Cl_2-Bzl). Lys was protected with 2-chlorobenzyloxycarbonyl (Cl-Z).

***P.f.* enolase:**	**ASEFYNSENKTYDLDFKTPNND**
Human α-enolase:	ASEFFRSG-K-YDLDFKSPD-D
Human β-enolase:	ASEFYRNG-K-YDLDFKSPD-D
Human γ-enolase:	ASEFYRDG-K-YDLDFKSPT-D

Figure 1. Comparison of amino acid sequences of enolases. A potential antigen, Ala^{256}-Asp^{277} (AD22) in P. f. *enolase and the coresponding segments in Human enolases are shown.*

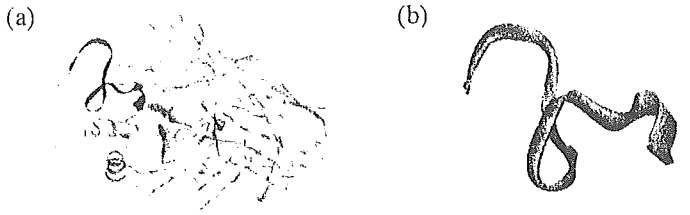

Figure 2. Calculated structure of (a) P.f. *enolase and (b) an antigenic segment (Ala^{256}-Asp^{277}, AD22).*

Scheme 1. The prepared sequence containing 28 amino acid residues (H-Glu_4-AD22-Gly_2-OH, (1)).

Glu-Glu-Glu-Glu-Ala-Ser-Glu-Phe-Tyr-Asn-Ser-Glu-Asn-
Lys-Thr-Tyr-Asp-Leu-Asp-Phe-Lys-Thr-Pro-Asn-Asn-Asp-
Gly-Gly

For the construction of **1**, protected peptide fragments were selected wherein Glu, Leu, Pro and Gly residues were placed at C-terminals, thereby minimizing the danger of racemization during the synthesis. These five fragments were prepared using the stepwise coupling procedure starting from Boc-Xaa-OH (Xaa = Leu, Glu(OBzl) and Pro) or H-Gly-OBzl. Then Boc-Xaa-OH was condensed with trichloroethanol (Tce-OH) to Boc-Xaa-OTce by using dicyclohexylcarbodiimide (DCC) in the presence of 0.1 equimolar of 4-dimethylaminopyridine (DMAP) to supress racemization. Boc group was deprotected by treatment with 4M HCl/dioxane or Trifluoroacetic acid (TFA). Condensation was performed by DCC or 1-ethyl-3-(dimethylaminopropyl)-carbodiimide hydrochloride (EDC.HCl)-hydroxybenzotriazole (HOBt) or 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)-HOBt.

Clude peptides were purified by silicagel, gel filtration chromatography and reprecipitation from appropriate solvents (such as, AcOEt, $CHCl_3$ and THF) by adding hexane. Tce group was deprotected by treatment with Zn/AcOH. Fragment condensation was performed by EDC.HCl-HOBt or O-(7-azabenzo-triazol-1-yl)-1,1,3,3,-tetramethyluronium Hexafluorophospate (HATU). Finally, the protected 28-residues peptide was deprotected by trifluoromethanesulfonic acid (TFMSA)-TFA-thioanisol. The final product was purified by gel-permeation

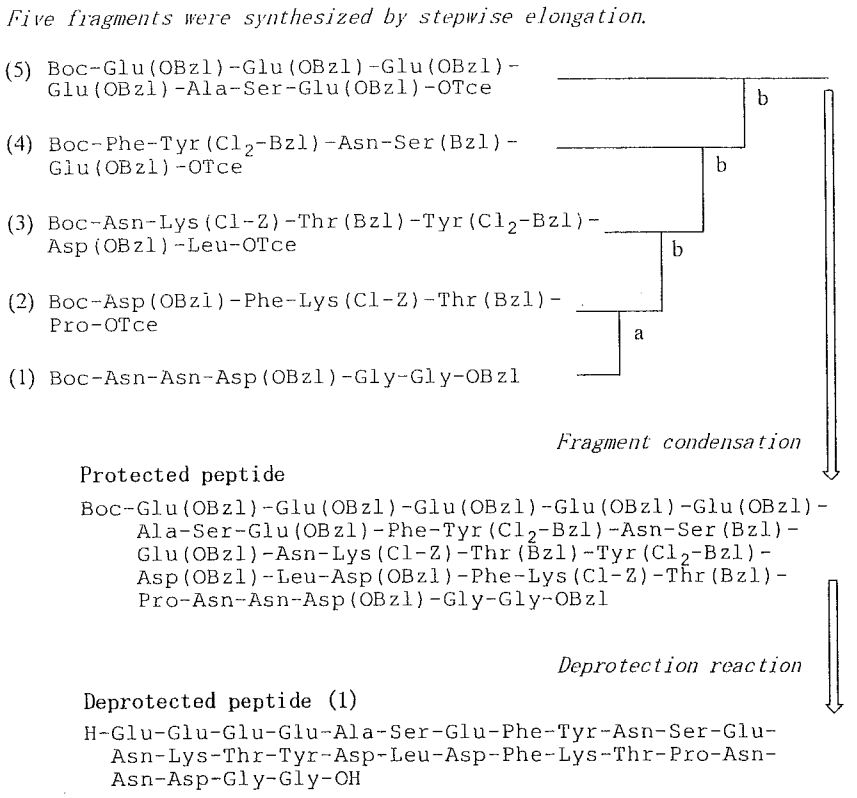

Figure 3. Synthesis of H-Glu$_4$-AD22-Gly$_2$-OH (1). Coupling route of segments are shown. Coupling reagents for segment condensation reactions: a, EDC.HCl-HOBt; b, HATU. All the condensations were performed in DMF. For the final deprotection reaction, the mixture of TFMSA-TFA-thioanisole-m-cresol was used.

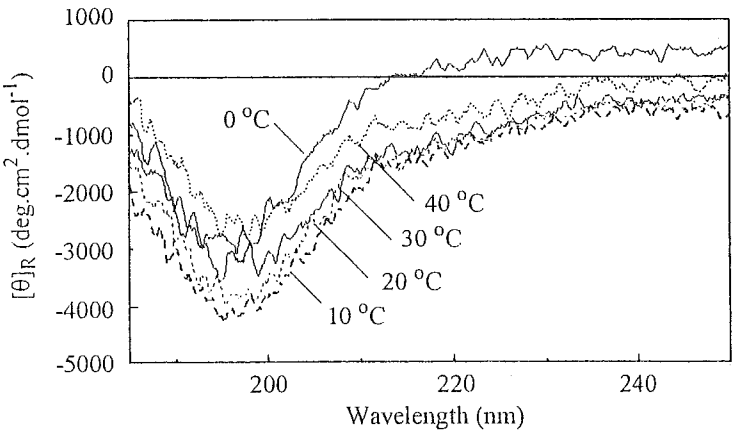

Figure 4. Variable temperature circular dichroism spectra of H-Glu$_4$-AD22-Gly$_2$-OH (1) in phosphate buffer (67 mM, pH 6.4), [1] = 1 mg / ml.

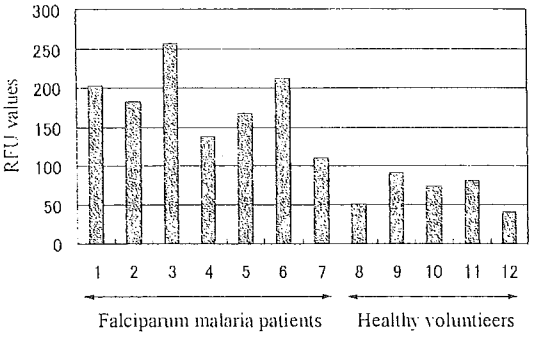

Figure 5. Fluorescence-ELISA Reactivity. The test was performed for the synthetic antigen, H-Glu$_4$-AD22-Gly$_2$-OH (1) (70 μL/well, [1] = 250 μg/mL in 0.05 M carbonate buffer at pH 9.8, reacted at 37°C) against sera from imported falciparum malaria cases in Japan (no. 1-7) and from healthy volunteers (no. 8-12). RFU values (= relative fluorescence unit) are plotted on the ordinate.

chromatography (Sephadex LH60) and HPLC. $[\alpha]_D^{20}$ = -21.3 (c 0.1, DMF); mp, 274-276°C; MALDI-TOF-MS, m/e = 3242.1 ($[M+H]^+$), 3224.1 ($[M+H-H_2O]^+$).

Reactivity of the synthetic peptide, H-Glu$_4$-AD22-Gly$_2$-OH (**1**) against sera from seven cases of imported *falciparum* malaria in Japan and from five healthy volunteers was tested using fluorescence-ELISA (Fig. 5). A considerable degree of the RFU values were measured for patients' sera (mean RFU = 181) compared with those of volunteers (mean RFU = 67).

In conclusion, the ELISA data have suggested that we have successfully synthesized the antigenic peptide suitable for the detection of falciparum malaria. We have expected the potential importance of the antigenic usage and the diagnostic application by further characterization and optimization of the synthetic peptide, **1**.

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IN VITRO SUSCEPTIBILITY AND GENETIC VARIATIONS FOR CHLOROQUINE AND MEFLOQUINE IN *PLASMODIUM FALCIPARUM* ISOLATES FROM THAI-MYANMAR BORDER

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Abstract. *In vitro* drug susceptibility to chloroquine (CQ) and mefloquine (MF) were assessed in 39 *P. falciparum* isolates from the Thai-Myanmar border area. To further characterize CQ- and MF-resistance profiles in this area, we also analyzed *pfcrt* K76T mutation that is critical for CQ resistance, and *pfmdr1* polymorphism that has an association with MF resistance. Eighteen isolates were successfully examined by *in vitro* tests for CQ, and 17 of them had resistance to the drug. Geometric mean concentration of CQ that inhibited the growth of parasites at 50% (IC_{50}) was 371 ± 227 nM (105-971 nM). Sixteen isolates were successfully examined by *in vitro* tests for MF, and 8 of them were resistant to the drug. Geometric mean of IC_{50} for MF was 41 ± 31 nM (4-125 nM). Genotypes of drug resistance, such as *pfcrt* and *pfmdr1* mutations, were also analyzed. All the 39 isolates had the same haplotype (CVIET) for PfCRT at its 72-76th amino acids. A *pfmdr1* Y86 mutation was found in 95% of isolates. A *pfmdr1* D1042 mutation was also present in 7 isolates, while no *pfmdr1* Y1246 mutation was observed. These results indicated a correlation between CQ resistance and the *pfcrt* T76 and *pfmdr1* Y86 mutations.

INTRODUCTION

The emergence of drug-resistant falciparum malaria is a serious threat to tropical countries. Chloroquine (CQ)-resistant *P. falciparum* was first reported in Southeast Asia in the 1950s and has since become widespread in this region (Looareesuwan and Chongsuphajaisiddlu, 1994; White, 1998; Breman, 2001). Recently, multi-drug resistant falciparum malaria has also become widespread in Southeast Asia, especially in the Thai-Myanmar border areas (Nosten *et al*, 1991; Peters, 1998); clinical efficacy of a number of drugs has been rapidly decreasing. Surveillance for drug-resistant malaria is based on strict *in vivo* criteria for treatment failure and on measurement of the *in vitro* susceptibilities of cultured parasites to antimalarials.

More recently, pathogen genotyping has proven to be useful in assessing resistance to some antimalarial drugs. Molecular methods, such as direct sequencing or restriction fragment-length polymorphism (RFLP) analysis, are currently used (Decuypere *et al*, 2003). Over the past two decades of using the polymerase chain reaction (PCR), numerous molecular markers for drug resistance of falciparum malaria parasites were described (Wongsrichanalai *et al*, 2002). Several genes attracted interest in the quest to elucidate polymorphisms related to antimalarial resistance and that could serve as specific molecular markers. There is consistent evidence that mutations in *pfcrt* (especially at position 76) correlate with *in vitro* and *in vivo* resistance of the parasite to CQ. There is also evidence that mutations in *pfmdr1* are associated with drug resistance (Wongsrichanalai *et al*, 2002). In this study, we investigated the *in vitro* drug susceptibility of CQ and mefloquine (MF), and *pfcrt* and *pfmdr1* mutations of *P. falciparum* isolates from 4 areas on the Thai-Myanmar border. This paper addressed *in vitro* drug resistance and concomitant gene mutations of parasite in the area where drug resistant malaria was reported to be highly endemic.

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MATERIALS AND METHODS

Study samples

The study was conducted at the Hospital for Tropical Medicine, Faculty of Tropical Medicine, Mahidol University, Bangkok. Isolates of *P. falciparum* were obtained from 39 symptomatic patients admitted to the hospital. These patients had been residing in four different areas of Thailand where malaria was highly endemic: Suan Phueng (western part of central region), Kanchanaburi (western part of central region), Mae Hong Son (northern region), and Tak (northern region) (Fig 1). After confirmation of

the diagnosis, venous blood was collected from each patient in a tube coated with EDTA (Venoject vacuum tube, Terumo, Tokyo, Japan). The patients' guardians gave written consent to this study that was approved by the Ethics Committee of Mahidol University, and we enrolled them. This survey research also followed the ethical guidelines for epidemiological studies set down by the Japanese Ministry of Education, Culture, Sports, Science and Technology; and Ministry of Health, Labor and Welfare.

In vitro drug susceptibility test

The *in vitro* drug susceptibility test used in this study was a modified semi-micro test described previously (Hatabu *et al*, 2003). Briefly, blood samples were washed three times with RPMI 1640 and re-suspended in RPMI 1640 (GIBCO BRL), pH 7.4, supplemented with 10% human serum (from non-immune Japanese donors without a previous history of malaria), and 25 μg/ml gentamicin (Sigma), 25 mM HEPES, and sodium bicarbonate, at a hematocrit of 5%. Five hundred microliters of the erythrocyte suspension were placed in each well of a tissue culture plate (24-well flat bottom, Corning Costar, New York, NY). Twenty microliters of chloroquine diphosphate or MF was added to each well (for CQ to create a series of 2 × dilutions, from 20 to 10,240 nM; and for MF to create a series of 10 × dilutions, from 0.01 to 1,000 nM). To monitor parasite growth, six wells per plate served as controls without antimalarials. Cultivation of parasites was done using the AnaeroPack® system (Mizuno *et al*, 2000). The AnaeroPack® CO_2 (Mitsubishi Gas, Tokyo, Japan) is a foil-packed paper sachet that, on exposure to air, immediately absorbs atmospheric O_2 and simultaneously generates CO_2 until a condition of 15% O_2 and 5% CO_2 is attained. The microaerophilic atmosphere produced within a sealed jar (AnaeroPack® Kakugata jar, SUGIYAMA-GEN, Tokyo, Japan) can be maintained for at least 24 hours. A portable thermostat incubator (SUGIYAMA-GEN) was carried to the laboratory, and the temperature inside the incubator was adjusted to 37°C. During *P. falciparum* cultivation, the sachet inside the jar was replaced with a new sachet every day when the culture medium was changed. When the schizonts were fully grown in the control wells, the culture plate was removed from the incubator. Thin-smear specimens, stained with Giemsa solution, were made from each well. We first counted the number of erythrocytes microscopically in the control smears until we encountered 50 schizonts. The effect of antimalarials on parasite growth was evaluated by observing the decreased number of schizonts per equal number of erythrocytes counted previously in the control cultures. The percentage of growth inhibition effect was calculated as follows: test well schizont count/control well schizont count (50) × 100.

DNA extraction

Fresh venous blood (0.5 ml) was blotted onto filter paper (Watmann) and dried. The filter paper was kept at room temperature until use. DNA was extracted from blots according to the method of Sakihama *et al*, (2001). Immediately, the dried filter paper was cut into 2 × 2 mm pieces and put into 1.5 ml tubes. Each blotted paper was incubated in 1 ml of Hepes-buffered saline (HBS), containing 0.5% (w/v) saponin (Sigma-Aldrich, St Louis, MO), at room temperature for 90 minutes, and washed twice with 1 ml of HBS. DNA remaining on the filter paper was isolated using a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacture's instruction. The eluted DNA was stored at 4°C until use.

Polymerase chain reaction (PCR)

The PCR analysis was performed using published methods for *pfcrt* (Lopes *et al*, 2002) and *pfmdr1* (Contreras *et al*, 2002). DNA fragments were amplified by PCR in 25-μl reaction mixture containing

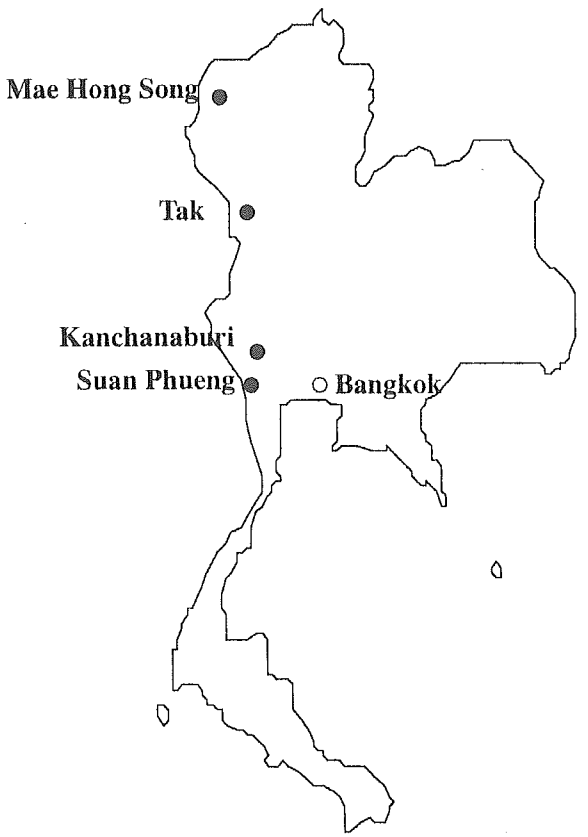

Fig 1- A map of Thailand showing (black circles) sampling areas.

2.0 μM of each primer, 250 μM dNTPs, 10 × PCR buffer (Invitrogen), 2.5 mM $MgCl_2$, and 1.0 units of Taq polymerase (Invitrogen). All the primers used in sequences and concomitant PCR conditions are shown in Table 1.

Restriction fragment length polymorphism and nucleotide sequencing of *pfcrt*

Restriction enzyme digestions of *pfmdr1* PCR products were carried out as previously described using the restriction enzymes *Afl* III (New England Biolabs, Beverly, MA), *Bgl* II (TAKARA Bio, Shiga, Japan), and *Vsp* I (MBI Fementas, Vilnius, Lithuania). The enzyme *Apo* I (New England Biolabs) was used to digest *pfcrt* PCR products. All fragments were subjected to gel electrophoresis on 2.0% agarose gels containing 0.5 μg/ml ethidium bromide.

Direct sequencing of codons 72 to 76 of *pfcrt* was attempted for all samples. The 206 bp PCR products were purified using QIAquick™ PCR purification system (QIAGEN) and sequenced by a BigDye™ Terminator Cycle Sequencing Ready Reaction kit (PE Biosystems) and the specific primers.

Data analysis

The drug concentration inhibiting parasite growth by 50% (IC_{50}) was calculated by the probit method (Inaba *et al*, 2001). Isolates with IC_{50} values for CQ more than 114 nM were regarded as resistant (Inaba *et al*, 2001). The threshold of the IC_{50} value for MF resistance was considered to be 40 nM (Price *et al*, 1999). Non-normally distributed data were described by median, range, and interquartile range (IQR); comparisons were made by Mann-Whitney *U* test. IC_{50} comparisons were made using Student's *t*-test or Welch's *t*-test. Statistical significance was assumed if the p-value was < 0.05.

RESULTS

***In vitro* drug susceptibility**

In total, 39 samples were analyzed for their *in vitro* drug susceptibility to CQ and MF. Eighteen of 39 samples were successfully examined for CQ. The geometric mean concentration of CQ that inhibited the growth of parasites at 50% (IC_{50}) was 371 ± 227 nM (105-971 nM). One of the 18 isolates was susceptible to CQ, and the IC_{50} value of this isolate was 105 nM. The highest IC_{50} to CQ (971 nM) was recorded for an isolate from Kanchanaburi. Isolates from the western part of the central region (Kanchanaburi and Suan Phueng) showed various IC_{50} values in the range between 105-971 nM, with a geometric mean (±SD) of 375 (±220) nM. Isolates from the northern region (Tak and Mae Hong Song) had IC_{50} values of 149-706 nM, with a geometric mean (±SD) of 352 (±236) nM. The differences between IC_{50} values of these areas were not significant.

Sixteen of 39 samples were also successfully examined for MF. Eight of 16 isolates (50%) were

Table 1
Polymerase chain reaction for amplification of fragments containing *pfcrt* and *pfmdr1* gene polymorphisms.

Primer	Sequence (5′ to 3′)	PCR program
pfcrt 76		
TCR-PA	ATGGCTCACGTTTAGGTGGAG	92°C, 30 sec; 45°C, 30 sec; 65°C, 45 sec;
TCR-P2	CGGATGTTACAAAACTATAGT	45 cycles
pfmdr1 86		
MDR-A	TTGAACAAAAAAGAGTACCGCTG	92°C, 30 sec; 45°C, 30 sec; 65°C, 45 sec;
MDR-B	TCGTACCAATTCCTGAACTCAC	45 cycles
pfmdr1 1042		
1042F	TATGTCAAGCGGAGTTTTTGC	94°C, 30 sec; 50°C, 30 sec; 68°C, 60 sec;
1042R	TCTGAATCTCCTTTTAAGGAC	45 cycles
pfmdr1 1246		
1246A	ATGACAAATTTTCAAGATTA	92°C, 30 sec; 45°C, 30 sec; 65°C, 45 sec;
1246B	ACTAACACGTTTAACATCTT	45 cycles

susceptible to MF. The geometric mean of the IC_{50} values was 41 ± 31 nM (4-125 nM). Six of 10 isolates (60%) from Kanchanaburi were MF-resistant. These isolates had IC_{50} values 10-125 nM, with a geometric mean (±SD) of 47 (±36) nM. Isolates from western and northwestern areas had IC_{50} values of 4-125 and 14-67 nM, with a geometric mean (±SD) of 41 (±32) and 41 (±27) nM, respectively. The statistical difference among IC_{50} values of these areas was not significant, either.

Of 16 CQ-resistant isolates, 8 (50%) were also resistant to MF. There was a significant positive correlation between the IC_{50} values of CQ and those of MF ($r^2 = 0.848$, $p < 0.0001$) (Fig 2).

pfcrt and *pfmdr1* polymorphisms in the isolates

pfcrt and *pfmdr1* polymorphisms were examined in 39 *P. falciparum*-positive samples (Fig 3). Mutations in both *pfcrt* and *pfmdr1* were quite common. RFLP analysis detected the presence of the mutant K76T allele in all samples. The CVIET sequence of codons 72 to 76 was found in all the 39 samples. N86Y and N1042D were present in 72% and 18% of the samples, respectively, while no polymorphism was found at position 1246.

Correlation between *pfcrt* and *pfmdr1* polymorphisms and *in vitro* antimalarial susceptibility

The prevalence of *pfmdr1* and *pfcrt* mutations in isolates that were successfully tested for drug susceptibility is presented in Table 2. The *pfcrt* K76T mutation was detected in all isolates. The N86Y mutation and mixed polymorphism (N86 and Y86) in the *pfmdr1* gene were also detected in 78% (14 of 18) and 22% (4 of 18) of isolates, respectively. The N1042D mutation was detected 28% (5 of 18) of isolates. However, a correlation between drug resistance and these mutations in *pfmdr1* was not found (Fig 4).

DISCUSSION

In Thailand, malaria continues to be a major public health problem due to the emergence of multidrug-resistant parasites. An increasing prevalence of *P. falciparum* resistance to CQ and MF have been reported. Our *in vitro* observations found CQ- and MF-resistant isolates to have been 94% and 50%, respectively, which indicated an alarmingly high prevalence of multidrug-resistant falciparum malaria on the Thai-Myanmar border.

Several studies have reported that the susceptibility of *P. falciparum* to CQ increased after the use of antimalarial was stopped for several years (Mita *et al*, 2003). Although mono-therapy with either CQ or MF for uncomplicated falciparum malaria has ceased for the past decades in Thailand, our results indicated that highly CQ-resistant falciparum malaria parasites were still prevalent in the present study areas. On Thai-Myanmar border, *P. vivax* is also highly prevalent, and CQ or CQ + primaquine is commonly used for the treatment of vivax malaria (Pukrittayakamee *et al*,

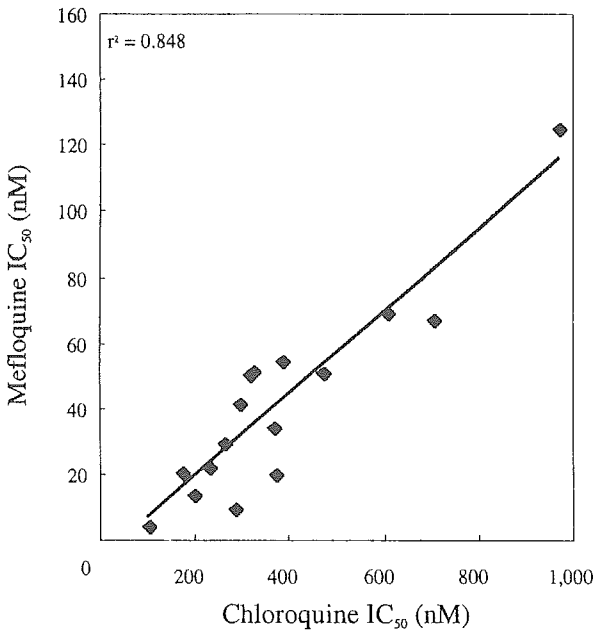

Fig 2- Linear regression analysis of the relationship between the IC_{50} values of chloroquine and mefloquine.

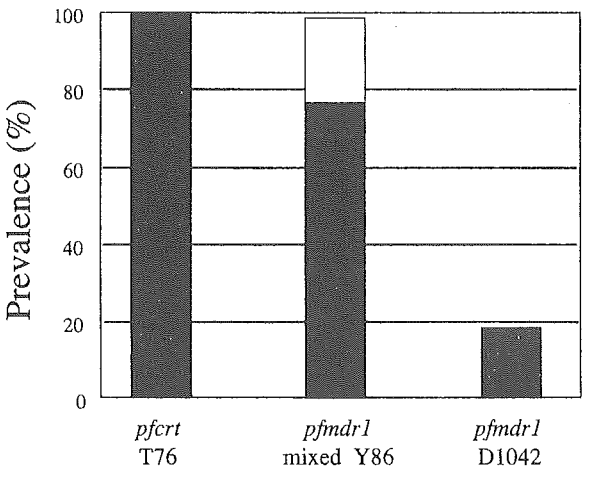

Fig 3- Prevalence of specific point mutations in *pfcrt* and *pfmdr1*. No mutations were observed at *pfmdr1* position 1246 (data not shown). Closed column indicated mutation in *pfcrt* and *pfmdr1*; open column indicated mixed polymorphism.

Table 2
pfcrt and *pfmdr1* mutations associated with chloroquine or mefloquine response *in vitro*.

Allele		Chloroquine *in vitro* (n=18)			Mefloquine *in vitro* (n=16)		
		Susceptible	Resistant	Total	Susceptible	Resistant	Total
pfcrt 76	Mutant (T)	1	17	18	8	8	16
	Wild (K)	-	-	-	-	-	-
pfmdr1 86	Mutant (Y)	1	13	14	6	6	12
	Wild (N)	-	-	-	-	-	-
	Mixed (N/Y)	-	4	4	2	2	4
pfmdr1 1042	Mutant (D)	-	5	5	4	1	5
	Wild (N)	1	11	12	4	7	11
	Mixed (N/D)	-	-	-	-	-	-
pfmdr1 1246	Mutant (Y)	-	-	-	-	-	-
	Wild (D)	1	17	18	8	8	16
	Mixed (D/Y)	-	-	-	-	-	-

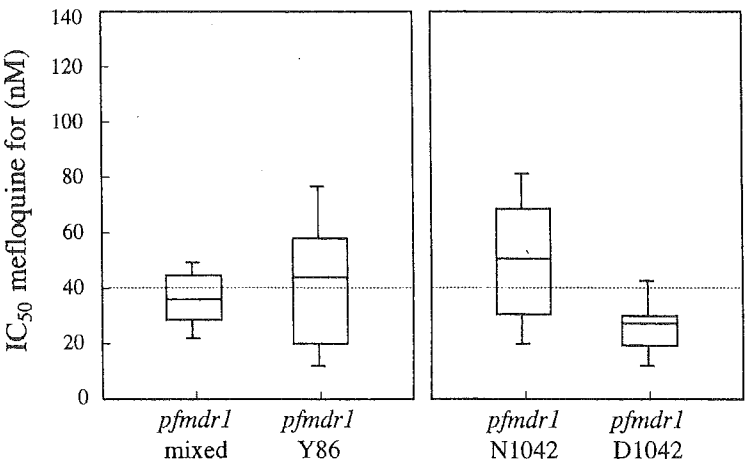

Fig 4- Box plots of median, range, and IQR according to *pfmdr1* mixed, Y86, *pfmdr1* N1042 and D1042 alleles.

2004; Konchom *et al*, 2003). Because a mixed infection of *P. falciparum/P. vivax* is commonly observed in the present study area, CQ used for *P. vivax* might be keeping pressure on the coexistent *P. falciparum*; thus affecting the recovery of CQ susceptibility.

The significant positive correlation between the IC_{50} values of CQ and MF was also observed in this study area, although several reports suggested an inverse relationship between CQ and MF resistance (Cowman *et al*, 1994; Ringwald *et al*, 1999). This may be attributed to dual drug pressures of CQ and MF in the highly endemic areas.

Although clinical study with adequate periods of patient follow-up is important for the assessment of optimal treatment of the drug resistant falciparum malaria, *in vivo* drug susceptibility testing is frequently difficult to implement. It is also difficult to perform parasite cultivation for *in vitro* drug susceptibility testing at hospitals or clinics in malaria endemic areas. In this situation, molecular genotyping of the isolates for an assessment of drug resistance can be an alternative means to indicate a need for a shift in antimalarial treatment regimens.

Recently, a strong association between mutant alleles of two genes (*pfcrt* and *pfmdr1*) and a high-level *in vitro* resistance to CQ in *P. falciparum* has been reported. Especially, a mutation in the *pfcrt* gene at