รายงานวิจัยฉบับสมบูรณ์

โครงการ การใช้ double-stranded RNA ในการยับยั้งการทำงานของยีน Histone deacetylase ของเชื้อ *Plasmodium falciparum*

โดย

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สัญญาเลขที่ RMU 4880044

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สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษากับสำนักงานกองทุนสนับสนุนการวิจัย

กิตติกรรมประกาศ

งานวิจัยนี้สำเร็จลุล่วงได้ด้วยดี โดยการสนับสนุนจากทุนเพิ่มขีดความสามารถด้านการวิจัยของอาจารย์รุ่นกลางในสถาบันอุดมศึกษา ตามโครงการความร่วมมือระหว่างสำนักงานคณะกรรมการการอุดมศึกษากับสำนักงานกองทุนสนับสนุนการวิจัย

ขอขอบคุณ ภาควิชาชีวเคมี ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ สถาบันอณูชีววิทยาและพันธุศาสตร์ บัณฑิตวิทยาลัย มหาวิทยาลัยมหิดล คณะแพทยศาสตร์ มหาวิทยาลัยธรรมศาสตร์ ศูนย์รังสิต ในการให้ความสนับสนุนแก่ คณะผู้ร่วมวิจัย

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Double-stranded RNA mediated gene silencing of histone deacetylase of *Plasmodium falciparum*

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Abstract

Acetylation and deacetylation of histones play an important role in transcription regulation, cell cycle progression and development events. The steady state status of histone acetylation is controlled by a dynamic equilibrium between competing histone acetylase and deacetylase (HDAC). Histone deactylase (HDAC) was recently suggested to be a potential new target for novel antimalarial compounds. The *Plasmodium falciparum* histone deacetylase 1(*Pf*HDAC-1) was recently cloned and sequenced. We have used long *Pf*HDAC-1 double-stranded RNA (dsRNA) to interfere with the cognate messenger expression and determined the effect on parasite growth and development. Chloroquine-and pyrimethamine-resistant *P. falciparum* K1 strain was exposed to dsRNA between 1-25 µg/ml cultures for 48 h and growth was determined by [^{3}H] hypoxanthine incorporation and microscopic assay. Parasite cultures treated with 10µg/ml *pf*HDAC-1 dsRNA exhibited 47 % growth inhibition when compared with either untreated control or culture treated with an unrelated dsRNA. *Pf*HDAC-1 dsRNA specifically blocked maturation of trophozoite to schizont stages and decreased *Pf*HDAC-1 transcript 44 % in treated trophozoites. These results indicate the potential role of this HDAC-1 as a target for development of novel antimalarials.

Key words:: *Plasmodium falciparum;* Malaria; Double-stranded RNA ;Histone deacetylase; *Pf*HDAC-1

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การใช้ double-stranded RNA ในการยับยั้งการทำงานของยีน Histone deacetylase ของเชื้อมาลาเรียชนิดฟัลซิพารัม

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บทคัดย่อ

ขบวนการ การเติมและการนำออกของหมู่ อะเซติล ของโปรตีน histone มีบทบาทสำคัญในการควบคุมและการพัฒนาของเซลล์โดย เอนไซม์ histone acetylase และ เอนไซม์ histone deacetylase เอนไซม์ hitstone deacetylase ของเชื้อมาลาเรียได้ถูกนำเสนอให้เป็นเป้าหมายในการศึกษาเพื่อพัฒนายาต้านมาลาเรียใหม่ ยีนของเอนไซม์ histone deacetylase ของเชื้อมาลาเรียชนิดฟัลซิพารัมได้ถูกศึกษาในระดับของยีนโดยทราบรหัสของเอนไซม์ทั้งหมด ในการศึกษาวิจัยนี้ได้ออกแบบและสังเคราะห์ double stranded RNA(ds) ที่มีลำดับเบสจำเพาะกับบางส่วนของยีน histone deacetylase ของเชื้อมาลาเรีย (*Pf*HDAC-1) เพื่อศึกษาว่าสามารถยับยั้งการสร้าง messenger RNA ของยีนดังกล่าวและผลต่อการเจริญเติบโตของเชื้อ โดยการเติม *pf*HDAC-1 dsRNA ในจานเพาะเลี้ยงเชื้อมาลาเรียชนิดฟัลซิพารัมสายพันธุ์ เค 1 ที่ดื้อต่อยา คลอโรควินและไพริเมทามีนในขนาด 1-25 ไมโครกรัม ต่อ 1 มิลลิลิตร ของ culture เป็นเวลา 48 ชั่วโมง และวัดการเจริญเติบโตและพัฒนาของเชื้อมาลาเรียโดยวิธี hypoxanthine incorporation และ การนับจำนวนของเชื้อโดยกล้องจุลทรรศน์ การทดลองพบว่า *pf*HDAC-1dsRNA 10 ไมโครกรัม ต่อ 1 มิลลิลิตร สามารถยับยั้งการเจริญเติบโตของเชื้อได้ร้อยละ 47 เมื่อเทียบกับเชื้อที่ไม่ได้รับสารหรือได้รับ dsRNA ของยีนที่ไม่เกี่ยวข้อง การยับยั้งการเจริญเติบโตของเชื้อมาลาเรียพบว่าเกิดขึ้นในระหว่างการพัฒนาของเชื้อจากระยะ trophozoite ไปยัง schizont พร้อมกับปริมาณของ messenger RNA ของยีน *pf*HDAC-1 ของเชื้อมาลาเรียลดลงร้อยละ 44 ผลการศึกษานี้บ่งชี้ถึงบทบาทสำคัญของเอนไซม์ histone deacetylase ของเชื้อมาลาเรีย ในการเป็นเป้าหมายสำคัญในการพัฒนายาใหม่ที่ใช้รักษาโรคมาลาเรีย

คำหลัก เชื้อมาลาเรียชนิดพลาสโมเดียม ฟัลซิพารัม; มาลาเรีย ; double stranded RNA ; เอนไซม์ histone deacetylase ; *Pf*HDAC-1

Introduction

Malaria is one of the most widespread of human parasitic diseases affecting approximately 300 million people annually worldwide and about 1.5-2.7 million people mostly children and pregnant women die every year from this disease [1,2]. Global efforts to eradicate this disease have failed due to the fact that the most virulent malarial parasite, *Plasmodium falciparum*, has become widely resistant to nearly all currently employed antimalarials and the development of an efficient vaccine is not that easy [2,3]. There is therefore, an urgent need for developing new classes of antimalarial compounds to combat this serious health problem disease.

The histones of *P. falciparum* have recently been studied as new targets for bloodstage antimalarial drug development [4,5]. Histones are nuclear core proteins, which are essential in packaging DNA into chromosomes. They are highly conserved across phyla, and may play a role in the pathology of malaria [5]. Acetylation and deacetylation post-translational modifications of histones play an important role in transcriptional regulation, cell cycle progression and development events in eukaryotes. The acetylation state of histones is controlled by a dynamic equilibrium activities of two familiy of enzymes histone acetyltransferase and the histone deacetylase(HDAC) [6,7]. A number of HDACs are present in human and can be classified into three categories; class I (HDAC 1,2,3,8), class II(HDAC 4,5,6,7,9,10) and class III (HDAC sirtulins). The HDACs from class I and II are zinc dependent mechanisms, but the ones from class III required NAD^+ for the activities [8]. Three histones from *P. falciparum* H2A, H2B and H3 have been sequenced and shown to have close homology with the histones from other organism [9]. Two histone deacetylase homologues from *P. falciparum, Pf*HDAC-1 and *Pf*Sir2 have recently

been cloned and sequenced [10,11]. The *Pf*HDAC-1 nucleotide sequence reveals an open reading frame of 1347 nucleotides which codes for a protein of 449 amino acid with the molecular weight of 51.4 kDa. The sequence is present as a single copy which shows significant homology to yeast, human and other eukaryotic HDACs and is predominantly expressed in the asexual blood stages and also in gametocytes [10]. Histone deacetylase inhibitors mostly in class I and II have been synthesized and found to have potent and specific anticancer activities and now currently in clinical trials and preclinical developments [12]. Apicidin, a cyclic tetrapeptide isolated from *Fusarium* spp. has been shown to inhibit the growth of malarial parasites by inhibiting histone deacetylase [4,13]. Other inhibitors of histone deacetylase, including SAHA, trichostatin, hydroxamate derivatives have also been shown to exhibit antimalarial effects with selectivities [13-15]. Recently attempts have also been made to modify and evaluate these inhibitors as potential antimalarial agents [9,15,16]. Thus, the inhibition of the plasmodial HDACs could be a possible approach for the development of novel and selective antimalarial therapy [4,15-17].

The completion of *P. falciparum* genome has provided a large amount of molecular information to study in more detail on the known proteins for their roles in the parasite's developmental stages and opportunity to discover new malarial drug targets to fight with the disease [17-19]. Moreover, the data also provide tool to determine structural differences of the potential targets with respect to their human homologues which can facilitate the development of novel and selective antimalarial therapy [9]. RNA interference (RNAi) has emerged as a powerful method for studying gene functions in a wide range of both unicellular and multicellular organisms and has been rapidly being applied to study the function of many genes

associated with human disease, in particular those associated with oncogenesis and infectious disease [20-22]. RNAi has also been studied in protozoan parasites including *Trypanosoma brucei* and *Plasmodium falciparum* [23-29]. In this study, we used long *pf*HDAC-*1* double-stranded RNA (dsRNA) to interfere with expression of the cognate messenger and to determine the ability of this dsRNA to inhibit growth and the development of asexual blood stage parasite in culture confirming the key role of this HDAC-1 enzyme in the intraerythrocytic stage of infection.

Materials and methods

*Parasite culture***.** *P. falciparum K1* strain (chloroquine- and pyrimethamine-resistant) isolated in 1979 from an infected individual in Kanchanaburi province, Thailand [30], was maintained in human erythrocytes in RPMI 1640medium supplemented with 10% human serum, 25mM Hepes, 32 mM $NaHCO_3$ under continuous culture *in vitro* using the candle-jar method of Trager and Jensen [31].

Preparation of P. falciparum genomic DNA. *P. falciparum* K1 strain cultures containing mostly late trophozoites were harvested when parasitemia reached approximately 15-20 %. The infected erythrocytes about 3 ml were washed with cold isotonic solution containing 0.9 % NaCl in 5 mM sodium phosphate buffer (PBS) pH 7.4. Parasites were liberated by incubating with 3 volume of 0.15 % saponin in PBS at 37° C for 10 min, and centrifuging at 5000 x g for 10 min. The intact parasites were then washed at least three times with cold isotonic buffer. DNA was extracted by alkali lysis method.

dsRNA preparation. Two DNA fragments coding for (349 nt) of *pf*HDAC-1 gene (GenBank accession number AF091326) were amplified by polymerase chain reaction from *P. falciparum* genomic DNA to be used as template for sense and antisense RNA. Two pairs of oligonucleotide primers with T7 RNA binding sites were used as follows: Sense strand template forward (5' T7-TCCTATGAAGCCTCAACG) and backward primers (5'AGCATGATGCAATCCTCC). Antisense strand template forward (5'T7-AGCATGATGCAATCCTCC) and backward primers (5' TCCTATGAAGCCTCAACG). The PCR products were purified and subjected to sequence analysis using the ABI Prism Dye Terminator Cycle sequencing Ready Reaction kit on a ABI Prism 377 DNA sequencer. The sense (sRNA) and antisense RNA (asRNA) were then generated using T7 RiboMax Express Large Scale RNA Production System (Promega). The dsRNA was prepared as follows: equal amounts of sRNA and asRNA were mixed and then heated at 75° C for 5 minute. Annealing was performed by slow cooling to room temperature for several hours. The dsRNA was analyzed and check for the quality on 1.5 % agarose gel. The unrelated dsRNA (GFP) was generated by using recombinant plasmid expressing stem loop GFP RNA as previously described [32].

In vitro assessment of antimalarial activity. The parasites were synchronized to the ring stage by sorbitol treatment [33]. A 200 µl aliquot of 1.5% cell suspension with 1–3% parasitemia was pre-exposed to 25 µl of the medium containing various concentration of dsRNAs between 1-25 µg/ml (4-100 nM), known HDAC inhibitor trichostatin (10nM), or buffer serum-free culture medium (for negative control), and with serial dilutions of chloroquine as positive control in 96-well tissue culture plate.

For hypoxanthine incorporation assay [34] , 0.5 μCi of [^{3}H]hypoxanthine (specific activity of 28.0 Ci/mmol, Amersham) in 25 μl medium was added to a 200 μl aliquot of 1.5% cell suspension with 1–1.5% parasitemia which was pre-exposed to 25 μl of the medium containing drugs for 24 h. After further incubation for 24 h, parasite DNA was harvested from each well onto filter paper (Whatman grade 934 AH) using an automated sample harvester. [^{3}H] Hypoxanthine incorporation in each well was determined in a Beckman liquid scintillation counter model LS-1801. Experiments were repeated twice. Data were reported as mean and standard deviation (SD) of percentage parasite growth inhibition in triplicate experiments relative to untreated control receiving medium alone without dsRNA or with unrelated dsRNA. Parallel parasite cultures were also used to prepare blood smears on microscopic glass slides and stained with Giemsa (Fisher, Pittsburg, PA). Parasitemia, parasite stage, and morphology of the cultures were determined by examination of 5000 red cells under oil immersion for the presence of intraerythrocytic *P. falciparum* and expressed as percent parasitemia. Results were expressed as the % inhibition of parasite growth as compared to control receiving medium alone. All values presented are the average of three experiments.

dsRNA treatment. For RNA extraction experiment, 25 μg of dsRNA was added per milliliter of highly synchronized ring stage parasites (5 % parasitemia, 4 % hematocrit) for 24 h. Control and unrelated dsRNA treated cultures were carried out at the same time. The infected cells were washed with cold RNase free buffer and centrifuged at 2,000 rpm at 4^{o}C for 10 min. Parasites were liberated by incubating with 5 volumes of 0.15% (w/v) saponin in PBS at 37^{o}C for 10 min and sedimented by

centrifugation at 4^{o}C, 5000xg for 10 min. The intact parasites were then washed at least three times with RNase free PBS.

RT-PCR assay. Total RNA was extracted from 3 ml treated cultures using TRI reagent. One µg of RNA was treated with RNase free DNase I (Qiagen) and purified using RNeasy column with the Qiagen clean-up procedure to avoid DNA contamination. Synthesis of cDNA was performed using random hexamers with the ImProm-II reverse transcriptase system (Promega). PCR was carried out with 2-4 µl of cDNA sample (or the negative control) in the presence of 0.5 µM primers; 18 S RNA forward (5′ CATTCG TATTCAGATGTCAGAGGTG) and backward primers (5′ CGTTCGTTA TCGGAATTAACCAGAC) or *pf*HDAC-1 forward (5′ CCAGATGTGTAGAACACG) and backward primers (5′ ACACCTGGAGCATGTTCTATGTGTC), 200 µM dNTP, and two units of Taq polymerase enzyme. After denaturation at 94 ° C for two minutes, 30 cycles with annealing at 52 ° C, elongation at 72° C and denaturation at 94 ° C were performed to amplify the *pf*HDAC-1 fragments. Eighteen cycles of the same amplification profile were used to amplify the ribosomal 18 S subunit. Under these conditions and number of cycles, the magnitude of the signals remained proportional to RNA concentrations. Fragments of expected lengths (482 bp, 298 bp for 18 S and *pf*HDAC-1, respectively) were observed by 1.5% agarose gel electrophoresis and relative amounts of mRNA were determined by densitometry .

Results and discussion

Inhibition of parasite growth in the stage of parasite maturation from trophozoite to schizont by pfHDAC-1 dsRNA

To demonstrate the antimalarial effect of *Pf*HDAC-1 dsRNA , parasite growth in cultures was measured in the presence of this 346 bp dsRNA targeting the coding region of parasite histone deacetylase gene or unrelated dsRNA (GFP) with similar size(~ 0.4 kb) or buffer alone using synchronous ring stage parasites after 48 h of exposure. In this assay, the parasites proceeded through their full life cycle from ring forms to the next generation of daughter rings. The long *pf*HDAC-1 dsRNA used in the study was prepared as described in material and method and analyzed on 1.5 % agarose gel as shown in Figure 1. The *Pf*HDAC-1 dsRNA of about expected size (346 bp) was predominantly in the preparation (lane 3). A summary of antimalarial activity for the tested dsRNAs by [^{3}H] hypoxanthine incorporation assay is presented in Figure 2. *Pf*HDAC-1 dsRNA at various concentrations was significantly reduced parasite growth (~ 47 % at 10 µg/ml (line 4)) when compared with either unrelated dsRNA (GFP) (11.7%) or with only medium alone (3%) (line 1) suggesting a sequence specific inhibition. The inhibition of parasite growth by this *pf*HDAC-1 dsRNA was somewhat dose dependent in the range of 1-10 µg/ml culture (line 1-5) and was saturated at higher concentration 25 µg/ml (line 6). The inhibitory effect of the parasite growth was also observed with a well known HDAC inhibitor trichostatin A at about 65 % at 10 nM (line7). Results from microscopic examination were in agreement with hypoxanthine incorporation assay (data not shown). These results show that using either *pf*HDAC-1 dsRNA or HDAC inhibitor affect parasite growth. However, increasing concentration of trichostatin A to 20 nM inhibited growth of parasite almost completely (data not shown) while increasing concentration of

*pf*HDAC-1 dsRNA could inhibit approximately only 50 % suggesting transient effect. The slightly inhibitory effect of unrelated dsRNA (GFP) which was observed in the experiment might be due to non specific phenomenon and sequence independent effect [32].

The malarial growth inhibition by dsRNAs corresponding to part of *P. falciparum* genes have been reported to be approximately 40-60 % which was comparable to our studies [25-29]. The amount of dsRNA used in these studies were in the range of 1-50 μg/ml parasite cultures. The difference in the concentration used might be due to the method of delivery of dsRNA to the malarial parasites. It has been shown that dsRNA against dihydroorotate dehydrogenase, an enzyme essential for the pyrimidine biosynthesis of *P. falciparum* could inhibit the growth of the parasite up to 60 % by electroporation of dsRNA at 1 μg/ml culture. No inhibition was observed when adding dsRNA to the medium without electroporation [25]. However, other studies have shown that adding dsRNAs against *P. falcipaurum* cysteine proteases or *P. falciparum* transcription factor *PfMyb1* directly to the medium at 25 μg/ml cultures could inhibit the growth of parasite about 30 % or at 48 % respectively [26]. Electroporation might enhance the delivery of dsRNA to parasites through at least three membrane layers: the red blood cell membrane, the parasitophorous vacuoular membrane, and the parasite plasma membrane. However, it has been observed that the parasitized erythrocytes could permit the entry of nucleic acid like oligonucleotides when adding directly to the medium while the uninfected ones could not, and approximately 0.1-0.15 % of dsRNA in the medium was taken up by parasites [26, 35,36].

To further explore the possible step at which *pf*HDAC-1 dsRNA operates during the developmental blood stage of parasite, assays were performed using *pf*HDAC-1 dsRNA at 10µg/ml added to ring stage parasites at the start of the experiment for 36 h. During this time parasites developed from ring to mature trophozoite and schizont which were continuously exposed to *pf*HDAC-1 dsRNA. This was devised to determine if the effect of dsRNA was on the maturation process or at invasion of merozoite to red blood cell. During the erythrocytic cycle, smears were performed every six hours and the number of parasites in trophozoite (Figure 3, line 1 and 2) and in schizont stage (Figure 3, line 3 and 4) were evaluated. The parasitemia observed in the trophozoite culture was similar in the control (Figure 3, line 1) and *pf*HDAC-1 dsRNA-treated cultures (Figure 3, line 2) , in contrast to that of schizonts showing a significant decrease in *pf*HDAC-1 dsRNA-treated parasites (Figure 3, line 4) when compared with the untreated control (Figure 3, line 3). The total inhibition was about 45 % which was comparable with that obtained from 48 h assay (Figure 2, line 5).This result suggests that parasite growth inhibition occur during the stage of parasite maturation from trophozoite to schizont transition.

Decreased expression of pfHDAC-1 mRNA in pfHDAC-1 dsRNA – treated culture

To determine whether the inhibition of parasite growth by *Pf*HDAC-1 dsRNA during maturation stage might be resulted from the interference of its cognate messenger, the expression of the *pf*HDAC-1 transcript was analyzed after 24 h treatment at the trophozoite stage, at the peak of its expression in the erythrocytic cycle by semi-quantitative reverse transcription (RT-PCR). The result of the RT-PCR evaluation of *pf*HDAC-1 transcript in cultures treated with *pf*HDAC-1 or unrelated

dsRNA (GFP), or left untreated is shown in Figure 4. The expression of the *pf*HDAC-1 transcript was decreased in *pf*HDAC-1 dsRNA-treated parasites (lane 6) when compared with untreated control parasites (lane 4) or with unrelated dsRNA (GFP) (lane 5). The expression of ribosomal 18s RNA gene was also conducted as an internal control and there was no change of 18s RNA transcripts in cultures after treatment with all tested dsRNAs or in the control (lane 1-3). After normalization against ribosomal 18s RNA, an approximately 44 % decrease in the *pf*HDAC-1 transcript was observed in *pf*HDAC-1 dsRNA-treated cultures when compared with control cultures or cultures treated with unrelated dsRNA(GFP). It has been established that the expression of *pf*HDAC-1 is at low level in the ring stage but at high level in both trophozoite and schizont together with its protein level [10]. Therefore, it is likely that *pf*HDAC-1 dsRNA added exogenously to culture during time at optimal levels of gene expression and enzyme production destroys its cognate messenger and thus inhibits parasite proliferation. This observation is similar to many studies previously reported that using long dsRNAs against *P. falciparum* genes encoding key enzymes influence the growth of parasites in culture with the decrease of their gene transcripts [25-29].

The mechanisms, by which gene expression is modified by dsRNA in *Plasmodium* are still not clearly defined. Since typical RNAi-associated genes have not yet been identified in *P. falciparum* genome, it is therefore suggested that the effect might be due to a different mechanism probably antisense effect rather than classical RNA interference [23,27,37,38] Interestingly, many antisense transcripts have been found during the erythrocytic cycles and have been suggested to be potential regulatory elements in gene transcription (39,40). It is possible that the

machinery required to process these antisense transcripts is present in *P. falciparum* and might be responsible for the resulting dsRNA. It is also possible that the essential factors in RNAi (dicer and RISCs) are transported into the intracellular parasites from human cells [37]. Whether which mechanisms is involved, data presented here show the susceptibility of *P. falciparum* to dsRNA against a parasite key enzyme involving in cell proliferation and differentiation histone deacetylase which has been shown to be a potential target in the development of new antimalarial agents.

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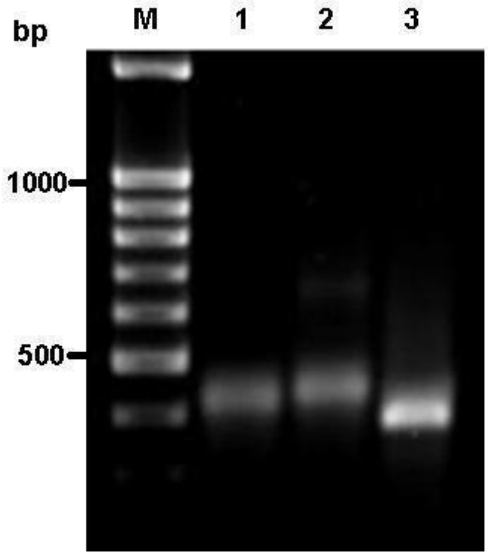

Figure 1. Agarose gel electrophoresis of single stranded RNAs and dsRNA of *Pf*HDAC-1 .To prepare dsRNA, antisense RNA(asRNA) and sense RNA(sRNA) were synthesized from DNA template using T7 RiboMax Express Large Scale RNA Production System (Promega). sRNA and asRNA were mixed, incubated at 75° C for 5 minutes and cooled slowly to room temperature for several hours to obtain its corresponding dsRNA, Lane 1, *Pf*HDAC-1 sRNA; lane 2, *Pf*HDAC-1 asRNA; lane 3, *Pf*HDAC-1 dsRNA. Lane (M) , a 100 bp DNA ladder marker

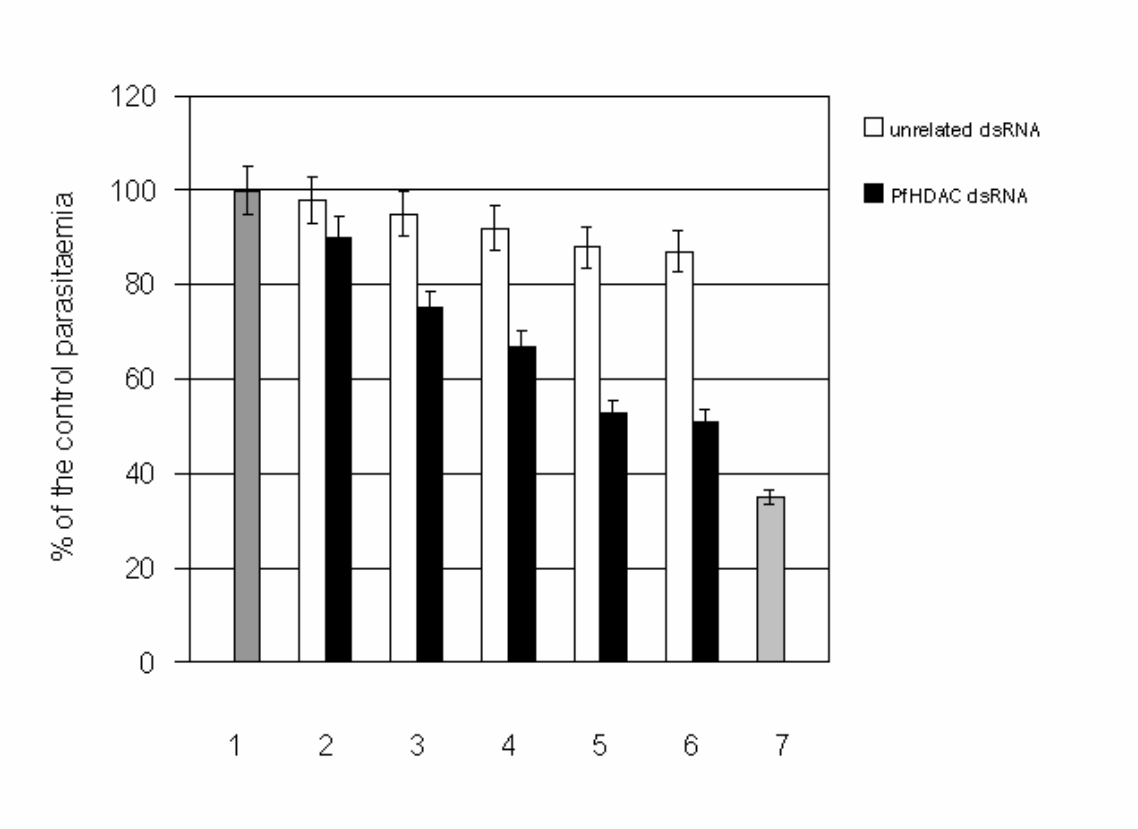

Figure 2: Effect of varying concentrations of *Pf*HDAC-1 dsRNA on the growth of *P. falciparum* in culture. *Pf*HDAC-1 dsRNA or unrelated dsRNA (GFP) at 0-25 µg/ml (line 1-6) was administered in triplicate parasite cultures at t = 0 to synchronized ring stage parasites. After 48 h incubation, the parasite growth was determined by [^{3}H]-hypoxanthine incorporation and the percent growth was calculated from average parasitemia in untreated controls. Line 1,(medium alone), line 2 (1 µg/ml), line 3 (3 µg/ml), line 4 (5 µg/ml), line 5 (10 µg/ml), line 6 (25 µg/ml), line 7, histone deacetylase inhibitor trichostatin A at 10 nM.

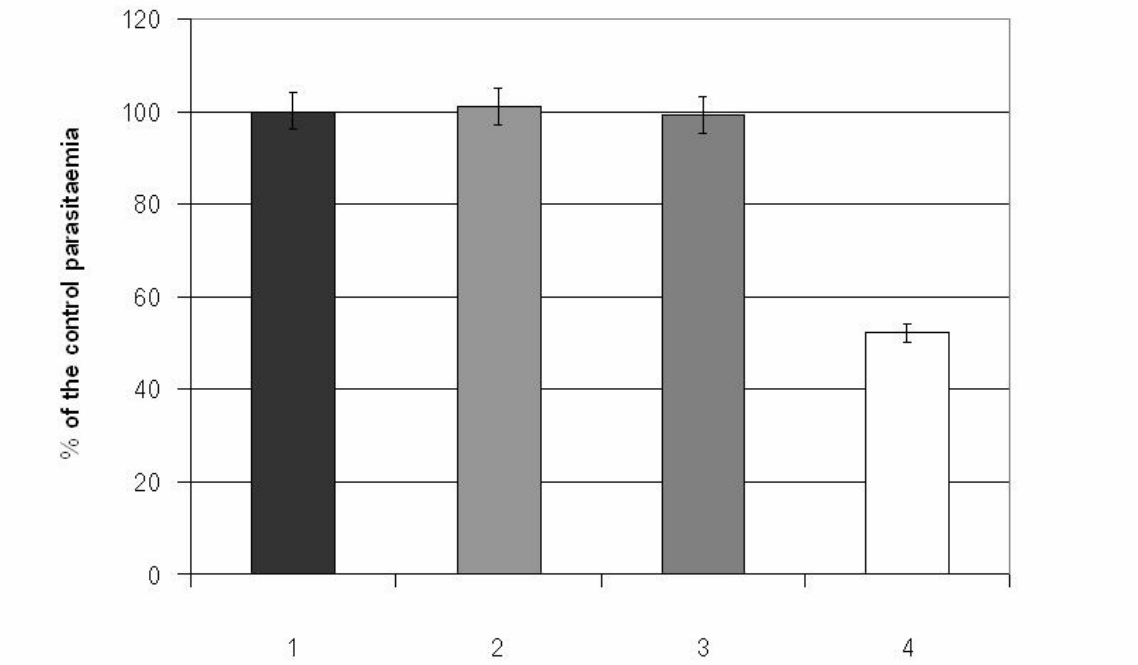

Figure 3. Effects of *Pf*HDAC-1 dsRNA against *P. falciparum* during parasite growth from ring to trophozoite and schizont. Parasitaemia of synchronized cultures was determined by microscopic assay at late trophozoite (line 1 and 2) and at late schizont stage (line 3 and 4) in untreated culture (line 1 and 3) and *Pf*HDAC-1 dsRNA treated culture (line 2 and 4). The experiment was repeated three times in triplicate. The results are expressed as a percentage of the control cultures.

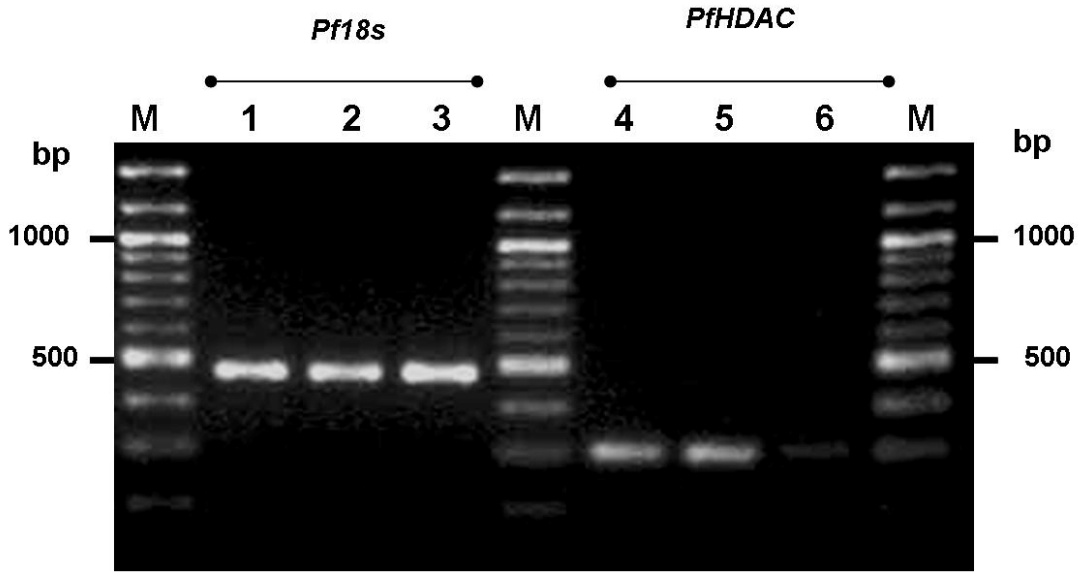

Figure 4. Analysis of *Pf*HDAC-1 mRNA expression by RT-PCR. Amplicon of 18 S ribosomal RNA(lane 1-3) , *Pf*HDAC-1 (lane 4-6) , were obtained after semi-quantitative RT-PCR on DNase-treated total RNA prepared from either untreated culture control (lane 1,4) unrelated dsRNA(GFP) (lane 2, 5) or *Pf*HDAC-1 dsRNA (lane 3, 6) treated culture and resolved on an ethidium bromide-agarose gel. The marker (lane M) is a 100 bp DNA ladder

Output:

1 ผลงานตีพิมพ์ในวารสารนานาชาติ

1.1 Florian Foger, Wilai Noonpakdee, Brigitta Loretz, Songwut Joojuntr, Willi Salvenmoser, Marlene Thaler, Andreas Bernkop-Schnurch. Inhibition of malarial topoisomerase II in Plasmodium faciparum by antisense nanoparticles. **Int. J. Pharmaceutics 319 (2006) 139-146**

1.2 W. Noonpakdee *, N. Sriwilaijaroen , S. Boonma' P. Attasart ,J. Pothikasikorn, S. Panyim. Inhibition of *Plasmodium falciparum* proliferation in vitro by double stranded RNA against malarial histone deacetylase gene . **Biochem. Biophys.Res.Commun. (Accepted for publication, Online Jan 29, 2009)**

1.3 Antimalarial effect of hydroxamate based histone deacetylase inhibitors: (Manuscript in preparation)

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2. Prof. Andreas Bernkop Schnurch, Institute of Pharmacy , U.of Innsbruck, Innsbruck, Austria

3. การนำเสนอผลงานวิจัย

3.1 Inhibition of *P. falciparum* proliferation by antisense and antisense nanoparticles against malarial topoisomerase II. W. Noonpakdee[1] F.Föger[2], and A. Bernkop-Schnürch[2]

Oral presentation: Abstract: The FEBS Journal 273, supplement 1, June 2006 p 66 31st FEBS Congress, **Istanbul, Turkey,** June 24-29. 2006

3.2 Wilai Noonpakdee , Nongluk Srivilaicharoen, Siriwan Boonma, Prapon Wilairat, Sakol Panyim.Double stranded RNA mediated gene silencing of histone deacetylase of *P. falciparum*

Poster presentation: Abstract : Gordon Research Conference on Malaria, Magdagen College, **U. of Oxford, UK,** September 9-14, 2007

3.3 W. Noonpakdee*[1], F.Föger[2], K. Suetrong,[1] A. Bernkop-Schnürch[2] Inhibition Of *P.falciparum* Proliferation *in vitro* by Antisense Nanoparticles against malarial Topoisomerase II

Oral Presentation: Abstract: Joint International Tropical Medicine Meeting 2007 "Health Security in the Tropics" 29-30 November 2007, Imperial Queen's Park Hotel, Bangkok, Thailand

3.4 Siriwan Boonma,Wilai Noonpakdee, Pongsopee Attasart, Wanchai Assvalapsakul,Prapon Wilairat and Sakol Panyim. Double-stranded RNA mediated gene silencing of topoisomerase II of *Plasmodium falciparum*.

Poster Presentation Award: Abstract; Joint International Tropical Medicine Meeting 2007 "Health Security in the Tropics" 29-30 November 2007, Imperial Queen's Park Hotel, Bangkok, Thailand

3.5 ศิริวรรณ บุญมา[1], วิไล หนุนภักดี[1],พงโสภี อัตตศาสตร์[2] ,วันชัย อัศวลาภสกุล[3], ประพนธ์ วิไลรัตน์[1]และ สกล พันธุ์ยิ้ม[1]DOUBLE-STRANDED RNA MEDIATED GENE SILENCING OF TOPOISOMERASE II OF *Plasmodium falciparum* การยับยั้งการแสดงออกของยีน topoisomerase II ใน *Plasmodium falciparum* ด้วย RNA สายคู่

Poster presentation: The 33 th Congress on Science and Technology of Thailand (STT33) October18-20, 2007, Walailak University, Nakhon Si Thammara

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Pharmaceutical Nanotechnology

Inhibition of malarial topoisomerase II in *Plasmodium falciparum* by antisense nanoparticles

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Abstract

New effective antimalarial agents are urgently needed due to increasing drug resistance of *Plasmodium falciparum*. Phosphorothioate antisense oligodeoxynucleotides (ODNs) silencing of malarial topoisomerase II gene have shown to possess promising features as anti malarial agents. In order to improve stability and to increase intracellular penetration, ODNs were complexed with the biodegradable polymer chitosan to form solid nanoparticles with an initial diameter of ~55 nm. The particle zetapotential depended on the chitosan/ODN mass ratio. Nanoparticles with mass ratio of 2:1 displayed a positive surface charge (+15 mV) whereas particles with 1:1 mass ratio were negatively charged (−20 mV). Additionally nanoparticles were found to protect ODNs from nuclease degradation. *P. falciparum* K1 strain was exposed to the chitosan/ODN-nanoparticles for 48 h in order to examine the effects of chitosan/antisense (AS) and chitosan/sense (S) oligodeoxynucleotide nanoparticles on malaria parasite growth. Both negatively and positively charged antisense nanoparticles as well as free antisense ODNs (in a final concentration of 0.5 μM) showed sequence specific inhibition compared with sense sequence controls. However, nanoparticles were much more sequence specific in their antisense effect than free ODNs. Nanoparticles with negative surface charge exhibited a significantly stronger inhibitory effect (~87% inhibition) on the parasite growth in comparison to the positive ones (~74% inhibition) or free ODNs (~68% inhibition). This is the first study demonstrating the susceptibility of *P. falciparum* to antisense nanoparticles.

Keywords: *Plasmodium falciparum*; Malaria; Nanotechnology; Chitosan nanoparticles; Antisense

1. Introduction

Malaria is one of the most prevalent human infectious diseases with up to 500 million infections occuring each year. *Plasmodium falciparum*, the most virulent of the four human malarial parasites, causes 1–3 million deaths each year, most of them among infants and young children in Africa. Both the broad collapse of preventive efforts and the decreased efficacy of current antimalarial drugs account for the global resurgence of malaria (Baird, 2005). New classes of antimalarial drugs are urgently needed due to increased resistance of *P. falciparum* against most currently used antimalarials (Noonpakdee et al., 2003). In this regard, use of antisense (AS) oligodeoxynucleotides (ODNs) offers an interesting alternative to traditional antimalarial drugs. The aim of the antisense approach is to interfere with gene expression by preventing the translation of proteins from mRNA (Lambert et al., 2001). The selectivity and flexibility of the antisense technology makes it an attractive strategy in treating several diseases. Phosphorothioate ODNs have been shown to inhibit HIV in vitro by a sequence specific and non-specific mechanism (Shaw et al., 1991). Similar to these findings several antisense treatments in malaria have demonstrated sequence specific as well as non-sequence specific inhibitory effects (Noonpakdee et al., 2003; Wanidworanun et al., 1999). Presently phosphorothioate ODNs are the most widely used modification of ODNs. Vitravene®, the first FDA-approved antisense drug from Isis, belongs to this type of synthetic nucleic acid. Progress has been made through the design of

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chemically modified ODNs with improved stability in serum but ineffective transport is still a limiting factor for antisense therapy (Chen et al., 2005). In vivo studies showed that free ODNs disappear from plasma after a few minutes whereas ODNs incorporated into nanoparticles showed a delayed plasma clearance (De Smidt et al., 1991). Additionally, phosphorothioate oligonucleotides still remain a substrate for nucleases. Carrier systems, such as nanoparticles, present a possible approach to improve the delivery properties of antisense ODNs and, furthermore, the complexation of ODNs into nanoparticles, enhances stability against enzymatic degradation (Junghans et al., 2000). Chitosan, a biocompatible polysaccharide has been shown to effectively complex plasmid DNA and to protect DNA from nuclease degradation (Mao et al., 2000). As the malarial parasite divides rapidly after invasion of human erythrocytes, DNA replicating enzymes or their related genes in the parasites offer suitable key targets. DNA topoisomerase II that catalyzes changes in DNA topology, by cleaving and re-ligating both strands of the DNA double helix (Felix, 2001) has been reported to be a target of interest for antisense therapy (Noonpakdee et al., 2003). In this study 30mer antisense ODNs against malarial topoisomerase II gene have been incorporated in chitosan nanoparticles, in order to investigate the feasibility of applying nanotechnology against *P. falciparum*. Antisense and sense chitosan/ODN nanoparticles with negative as well as positive zeta potentials have been developed in order to investigate their release profile, stability against nuclease degradation, toxicity and their antimalarial effects against *P. falciparum* in vitro.

2. Materials and methods

2.1. Materials

Phosphorothioate oligodeoxynucleotides (ODNs) were designed to be antisense or sense to sequence within the structural region of *P. falciparum* topoisomerase II gene as listed in Table 1 (Noonpakdee et al., 2003). All phosphorothioate-ODNs were synthesized by VBC Biotech (Vienna, Austria) and were of HPLC grade. Chitosan (middle-viscous, medium molecular mass: 400 kDa; degree of deacetylation: 83–85%) was obtained from Fluka Chemie (Buchs, Switzerland). Giemsa staini was obtained from Fisher (Pittsburg, PA), RPMI 1640 medium, from Gibco, Triton X-100, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) and $NaHCO_3$ from Sigma (St. Louis, MO, USA). All other chemicals used were of analytical grade.

2.2. Sample preparation

Chitosan (1% w/v in 6% v/v CH_3COOH, 100 mL) was depolymerised with 10 mL of $NaNO_2$ solution (0.5% w/v) for 1 h. After 1 h agitation, the depolymerised chitosan was precipitated by addition of 4 M NaOH solution to obtain pH 9, filtered and washed twice with acetone (Huang et al., 2004). The solid residue was resuspended in 20 mL 0.1% v/v CH_3COOH, dialysed twice against 4 L double-distilled water (24 h) and lyophilised. Phosphorothioate oligodeoxynucleotides were complexed with depolymerised chitosan with mass ratios (chitosan/ODNs) of 1:1 and 2:1 by mixing aliquots of 100 μg of chitosan, dissolved in 500 μL of 0.025% acetic acid (pH 5.5 adjusted with 1 M NaOH) with solutions of 50 or 100 μg of ODNs, dissolved in 500 μL of double-distilled sterile filtered water. Both solutions were preheated separately to 50 °C with a thermo mixer for 15 min. Then 500 μL of the 0.02% w/v chitosan solution was added to the ODNs solution followed by intensive vortexing for 90 s.

2.3. Size determination of the particles

The hydrodynamic diameters of the nanoparticles were measured by photon correlation spectroscopy (PCS) using a PSS NICOMP™ 380 DLS/ZLS (Santa Barbara, California, USA) with a 7.5 mW laser diode at 635 nm. Size determination of the nanoparticles was carried out in double-distilled water and additionally in RPMI 1640 medium including 10% v/v sterile filtered human serum (incubated for 30 min at 37 °C). Filtration of serum was performed in order to remove cellular components which might influence PCS measurements. The measurements were carried out at room temperature with a scattering angle of 90 °C. To verify the results obtained by PCS, transmission electron microscopy (TEM) of the nanoparticles in absence as well as in presence of 10% v/v sterile filtered human serum was carried out. A drop of approximately 10 μL of each suspension was mounted on pioloform-coated copper grid. Negative staining was carried out with 1% (w/v) uranyl acetate to enhance contrast. For samples with a high density of particles, suspensions were diluted with an equal volume of 1% uranyl acetate and mounted on grids. In addition pure chitosan ODN nanoparticles were mounted without negative stain to avoid artefacts by chemical influence. Specimens were examined with a ZEISS LIBRA 120 digital energy filter transmission electron microscope (EFTEM). Elastic imaging with the in-column omega filter (0 eV) and inelastic imaging with a selected energy loss of 50–130 eV were applied.

Table 1
Sizes and zetapotentials of particles formed by mixing chitosan with 30mer ODNs at 1:1 and 2:1 mass ratios measured as hydrodynamic diameters by PCS

Mass ratio (Chitosan:ODN)	Particle size (nm)		Zetapotential (mV)	
	Gauss number in distilled water	Gauss number in culture medium + 10% serum	Gauss number in distilled water	Gauss number in culture medium + 10% serum
1:1 Antisense	58 ± 25	58 ± 35	−20.7	−2.5
1:1 Sense	51 ± 21	56 ± 32	−19.0	−3.0
2:1 Antisense	55 ± 19	54 ± 39	+14.4	−1.9
2:1 Sense	55 ± 18	56 ± 38	+16.0	−2.0

Microphotography and documentation were performed using a VarioSpeed SSCCD camera (BM-2k-120). For size measurement and also for documentation an analySIS Pro TEM software and Adobe photoshop were used.

2.4. Electrokinetic potential

The zeta potentials of the nanoparticles were measured in double-distilled water and additionally in RPMI 1640 medium including 10% sterile filtered human serum (incubated for 30 min at 37 °C). The zeta potential was analysed by measuring the electrophoretic mobility using a PSS NICOMP™ 380 DLS/ZLS. All measurements were carried out at room temperature.

2.5. Oligonucleotide loading and release

The amount of ODNs complexed in the chitosan nanoparticles was determined by agarose gel-electrophoresis and by HPLC. Electrophoresis was performed in 2% agarose gel in 1 × Tris/acetic acid/EDTA buffer (pH 7.4) containing 500 μg/L of ethidium bromide at 30 V for 1 h. Gels were visualized under UV light. In order to evaluate the amount of released ODNs under physiologically buffered solution, nanoparticles were complexed with chitosan in the mass ratios 1:1 and 2:1 as described above and then diluted 1:1 with 2 × phosphate buffer saline (PBS) (pH 7.4). Nanoparticles were incubated at 37 °C and 400 rpm with a thermomixer. After 0, 0.5, 1, 2, 6, 24, 48 h, samples were removed, centrifuged at 29,700 × *g* for 60 min (Sigma 3–18 K centrifuge) and 30 μL of the supernatant was analyzed by a weak-base anion exchange HPLC assay, using a PRP-X600 Anion Exchange 4.6 × 100 HPLC column (Hamilton, Reno, Nevada, USA). A two-eluent system, with eluent A consisting of 80:20 100 mM Tris, pH 8.0:acetonitrile and eluent B consisting of 80:20 100 mM Tris, 2.5 M LiCl, pH 8.0:acetonitrile was used. A linear gradient from 100% eluent A to 100% eluent B in 15 min at a flow rate of 2 mL/min was performed. The amounts of ODNs were determined by measuring absorbance at 260 nm.

2.6. Protection of ODNs against digestion in plasma

Digestion of oligonucleotides was determined by incubating 50 μL of free or complexed ODNs in RPMI 1640 medium containing heat or non-heat inactivated human plasma at a final concentration of 20% at 37 °C and 400 rpm (Eppendorf Thermomixer comfort). After 0, 0.5, 2, 4 and 8 h, digestion was terminated with 50 μL of 0.5 M EDTA per 200 μL of samples. The chitosan/ODN particles were then dissolved by the addition of 100 μL of 5 M sodium chloride and incubation for 24 h. The amount of non-degraded ODNs were analysed by HPLC as described.

2.7. Evaluation of red blood cell lysis

Red blood cell lysis test was performed as described previously (Guggi et al., 2004). In brief, blood was obtained from male human (blood group 0+) and erythrocytes were collected by centrifuging (1500 × *g*, 5 min, 10 °C; SIGMA 3–16 K centrifuge) and washing in a washing solution (17.5 g sorbitol and 0.8 g NaCl in 100 mL double distilled water) for four times. A 2% v/v erythrocyte solution was prepared by resuspending the final cell pellet in an appropriate volume of washing solution. Aliquots of 400 μL of the erythrocyte solution were transferred into each well of a 24-well plate. Then 50 μL of the antisense nanoparticles with mass ratio of 1:1 and 2:1, free ODNs or corresponding controls were added and the suspensions incubated for 3 h at 37 °C. The final concentration of ODNs was 0.5 μM. After incubation, the samples were centrifuged and the supernatants (400 μL) analyzed for haemoglobin release by measuring the absorbance at 570 nm (UV-1202 SHIMADZU spectrophotometer). A 50 μL aliquot of washing solution was used as negative control, and positive control (100% haemoglobin release) was a Triton X-100 detergent (5% v/v) lysed solution. Results were expressed as the amounts of haemoglobin released caused by the test compounds as percent of the total amount. Furthermore the same experiments were repeated in the presence of 10% human plasma.

2.8. Growth inhibition of P. falciparum

P. falciparum K1 strain isolated in 1979 from an infected individual in Kanchanburi province, Thailand, was maintained in human erythrocytes in RPMI 1640 medium supplemented with 10% human serum, 25 mM Hepes, 32 mM $NaHCO_3$ under continuous culture using the candle-jar method of Trager and Jensen (1976). The parasites were synchronized to the ring stage by repeated sorbitol treatment (Lambros and Vanderberg, 1979). A 200 μL aliquot of 2% v/v cell suspension with 2% parasetemia was pre-exposed to 25 μL of the medium, containing the test compounds or serum-free culture medium for negative control, in 96-well culture plates. All ODN containing test compounds have been evaluated in final ODN concentrations of 0.5 and 1 μM. Additionally, free chitosan in a final concentration of 0.001% w/v was tested, in order to exclude an unspecific inhibitory effect. After 48 h incubation at 37 °C under candle jar condition, supernatant from each well was removed. One drop of the residue was used to prepare thin blood smears on microscopic glass slides and stained with Giemsa. Parasitemia (the number of parasites per 100 red blood cells), parasite stages and morphology of the cultures were determined by microscopic examination by counting of 5000 erythrocytes under oil immersion. Results were expressed as the percent reduction of parasite growth as compared to the control receiving serum-free medium alone without ODNs. All results presented were the average of at least three independent experiments in triplicate. Statistical significance between average percentage reduction in parasite growth compared with control was conducted using Student's *t*-test.

3. Results

3.1. Particle size and surface charge

The hydrodynamic diameters of antisense and sense ODNs complexed with chitosan at mass ratio of 1:1 and 2:1 were

measured by PCS (Table 1). The measurements were carried out in double distilled sterile filtered water at room temperature. Chitosan was complexed with 30mer antisense and sense ODNs in the form of spherical particles with mean diameters of ~51–58 nm. Different amounts of chitosan did not noticeably affect the mean diameters of the particles. Also the sequence of the ODNs showed no influence on particle size. In addition, TEM of antisense nanoparticles of chitosan/ODN was performed to verify the results obtained by the PCS measurement and to determine the structure of the nanoparticles (Fig. 1A). The diameters of the antisense nanoparticles with 1:1 and 2:1 mass ratios ranged between ~30 and ~90 nm, which was in good agreement with the PCS measurements. After incubation in serum, particle size analysed by PCS did not change significantly (Table 1). This observation could be verified by TEM of nanoparticles in presence of human serum (Fig. 1B). No agglomeration between nanoparticles and serum components could be identified.

In addition, the surface charges of the nanoparticles were determined in double distilled water and in RPMI 1640 medium containing 10% human plasma. In distilled water, the antisense and sense nanoparticles with mass ratio of 1:1 were negatively charged. The zeta potential of the 1:1 antisense and sense nanoparticles was −20.7 and −19.0 mV, respectively. At the mass ratios of 2:1 of chitosan/ODN, nanoparticles were positively charged, +14.4 mV for antisense and +16.0 mV for sense nanoparticles.

On the other hand, after incubation of the nanoparticles in RPMI 1640 medium containing 10% human serum for 30 min at 37 °C, all particles showed slightly negative zeta potentials, ranging from −1.9 to −3.0 mV. Absorption of serum proteins on the charged nanoparticles and a shielding effect of ions from the culture medium could account for the decrease of the surface charge.

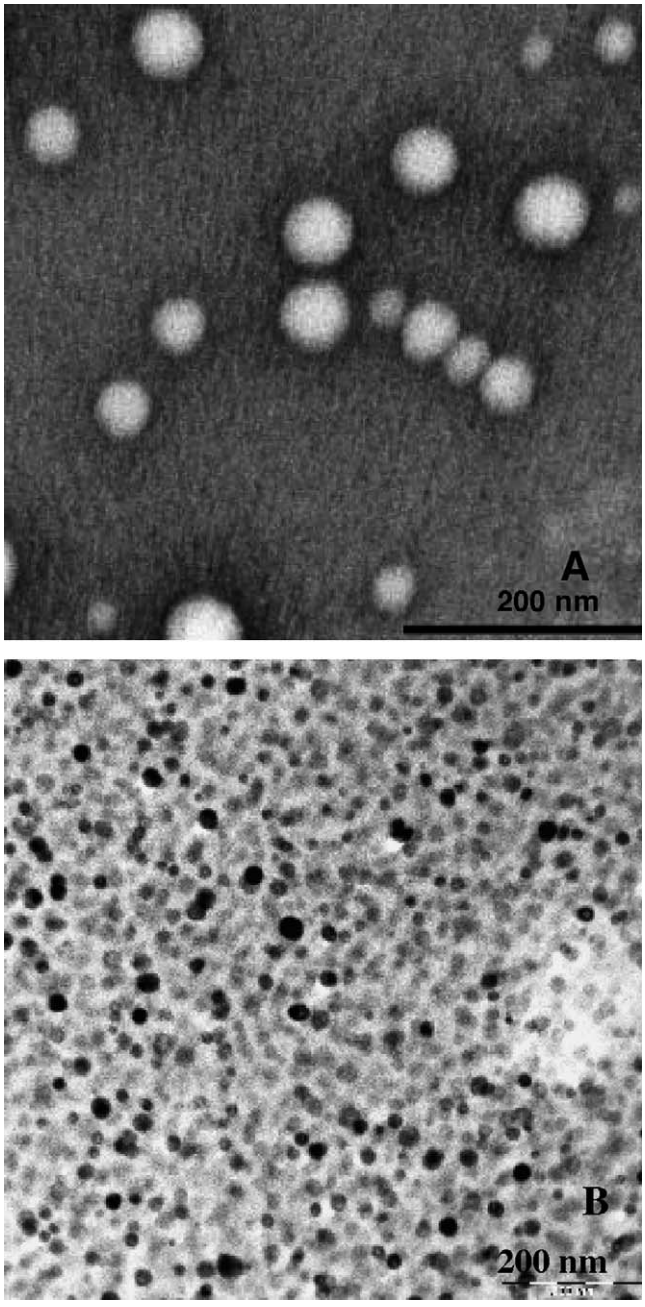

Fig. 1. Transmission electron micrographs of 2:1 nanoparticles in absence (A) (elastic imaging with 0 eV) and presence of 10% v/v human serum (B) (inelastic imaging with an energy loss of 50 eV).

3.2. Oligonucleotide loading and release

The amounts of ODNs complexed in the chitosan nanoparticles were determined by agarose gel-electrophoresis (Fig. 2). At mass ratio of 1:1, as well as 2:1, ODNs were complexed and bound to the nanoparticles. In the HPLC assay ~4% of unbound ODNs were found in solution for the 1:1 nanoparticles whereas no unbound ODNs were detected for the 2:1 particles. In order to determine the stability of the particles under physiological pH and salt conditions, the release of ODNs was investigated in PBS (pH 7.4) at 37 °C (Fig. 3). The 2:1 particles showed a minor release of ODNs. After 48 h, 6% of unbound ODNs were detected. Nanoparticles with 1:1 mass ratio showed a faster release. A 48% of uncomplexed ODNs were detected after 48 h.

3.3. Protection of ODNs against digestion in plasma

The protective effect of chitosan against degradation of oligonucleotides in non-heat inactivated human plasma was investigated. Antisense nanoparticles as well as free ODNs were

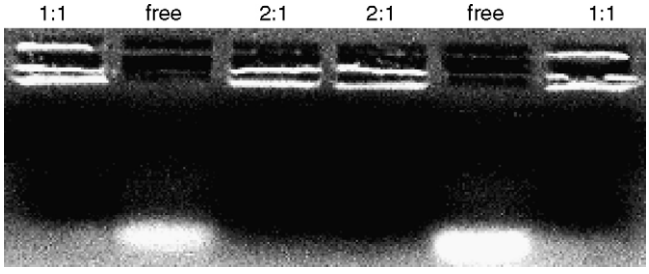

Fig. 2. Determination of the ODN content of the complexes. Gel electrophoresis of mixtures of chitosan with antisense and sense oligonucleotides in the mass ratios 1:1, free ODNs and 2:1 from the left line on. (agarose gel pH 7.4).

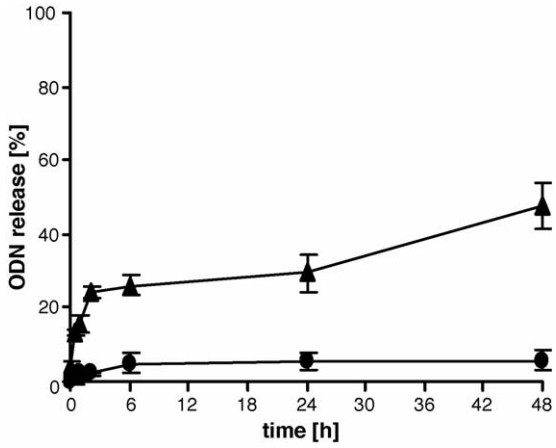

Fig. 3. Release of ODNs from particles in PBS. Particles in the mass ratio 1:1 (triangles) and 2:1 (balls).

incubated in RPMI 1640 culture medium containing human plasma in a final concentration of 20% at 37 °C. The amounts of undegraded ODNs were measured for 8 h (Fig. 4). Intact oligonucleotides were analysed by HPLC. After an initial phase of rapid degradation (∼17% in 0.5 h), digestion of unbound ODNs was decreased. Significant protection of ODNs degradation was demonstrated with both types of chitosan nanoparticles, but particularly for the 2:1 nanoparticles. After 2 h incubation, ∼99% of oligonucleotides remained intact for the 2:1 and ∼96% for the 1:1 nanoparticles. The small amounts of uncomplexed ODNs of the 1:1 nanoparticles, as visualized in the agarose gel, might have been degraded, explaining the small difference between the two mass ratios. In addition stability of ODNs and nanoparticles in heat inactivated plasma was evaluated and no degradation for all test compounds was detected still after 24 h (data not shown).

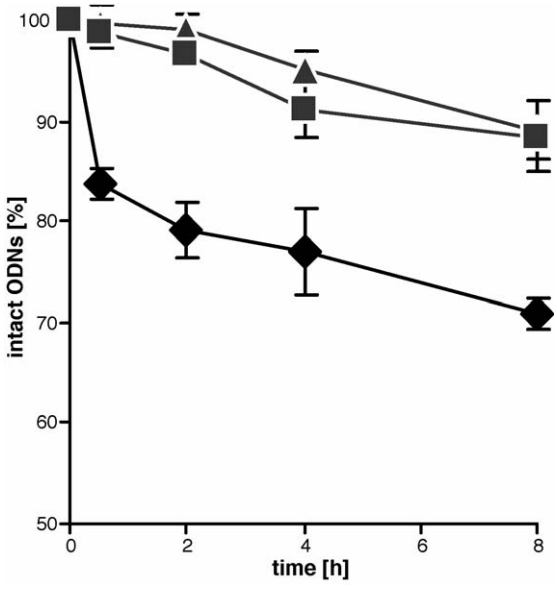

Fig. 4. Protection of oligonucleotides against degradation in cell culture medium containing not heat inactivated human plasma. Free ODNs (diamonds), nanoparticles in the 1:1 (squares) and 2:1 (triangles) mass ratio.

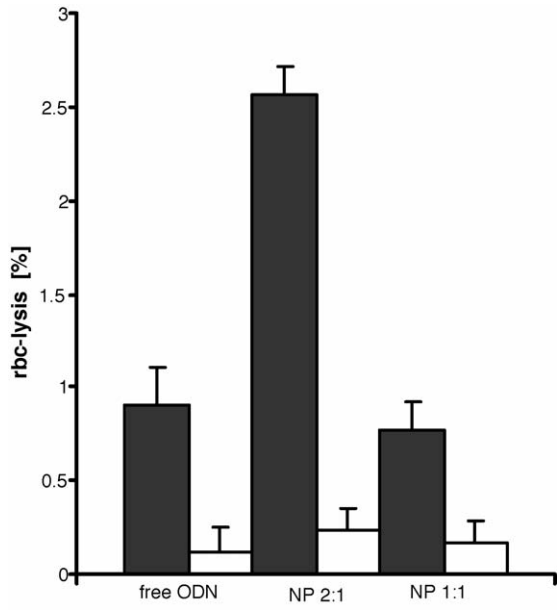

Fig. 5. Red blood cell (RBC) lysis in absence (black bars) and presence (white bars) of plasma.

3.4. Evaluation of red blood cell lysis

In this study haemolysis experiments were performed to investigate interactions of nanoparticles, with positive as well as with negative surface charge, with the negatively charged red blood cell membrane. The membrane damaging properties of the test compounds were determined by the quantification of released haemoglobin. Results are shown in Fig. 5. After 3 h of incubation, nanoparticles with positive zeta potential showed a higher membrane damaging effect causing a significantly higher haemoglobin release (2.6%) as compared to nanoparticles with negative surface charge (0.8%) or unbound ODNs (0.9%).

Furthermore the membrane damaging properties of the test compounds in the presence of 10% human plasma were investigated. Haemoglobin release was significantly lower for all test compounds (∼0.2%) in the presence of human plasma.

3.5. Growth inhibition of P. falciparum

The antimalarial effects of antisense nanoparticles with negative as well as positive zeta potential and free antisense ODNs were evaluated. *P. falciparum*, synchronized to ring stage, was exposed for 48 h to the samples. In control assays, parasites proceeded through their full life cycle from ring forms to trophozoite and schizont forms and invasion of red cells to produce the next generation of daughter rings. The antimalarial activity of the test compounds is presented in Fig. 6. All antisense phosphorothioate oligonucleotide-containing samples in a final concentration of 0.5 μM significantly reduced parasite growth compared to the sense sequence-containing control samples or with medium alone, suggesting sequence specific inhibition (Table 2). Highest inhibition of *P. falciparum*

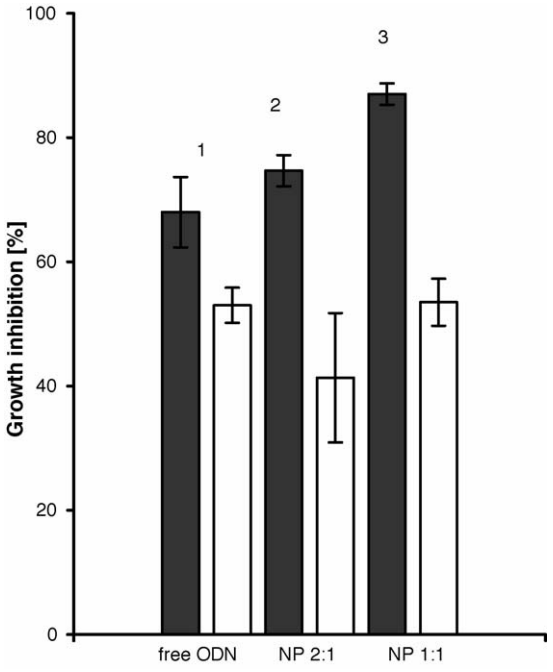

Fig. 6. Growth inhibition of *P. falciparum* with antisense (black bars) and sense (white bars) ODNs. Free ODNs, nanoparticles in the mass ratio 2:1 and 1:1 from the left line on. Indicated values are means ± S.D. of three experiments, each with triplicate samples; 1: differs from sense ODNs, $p < 0.015$; 2: differs from 2:1 sense complex, $p < 0.006$; 3: differs from 1:1 sense complex, $p < 0.0002$.

growth, approximately 87% reduction, was achieved with antisense nanoparticles with negative surface charge. Sense ODN nanoparticles with the same mass ratio reduced parasite growth by 53%. This difference clearly demonstrated sequence specific inhibition.

Antisense nanoparticles with the 2:1 mass ratio reduced parasite growth by ∼75% compared to 41% inhibition by sense nanoparticles, demonstrating that complete neutralization of the negative charge by complexation with positively charged chitosan did not prevent sequence specific inhibition of parasites. Free antisense ODNs reduced *P. falciparum* growth by 68% compared to 53% by sense oligonucleotides demonstrating lower significance of sequence-specific inhibition. In order to exclude an effect of pure chitosan on parasite growth, polymer without ODNs was added to the culture, but displayed no inhibition (data not shown). At a concentration of 1 μM, all ODNs-containing samples reduced parasite growth independent of the sequence (data not shown).

Table 2
Phosphorothioate oligodeoxynucleotides specific for the translation initiation sites and internal coding regions of *P. falciparum* topoisomerase II gene

Oligomers	Sequence	Nucleotide numbers
AS	ATG TAA TAT TCT TTT GAA CCA TAC GAT TCT	163-134
S	AGA ATC GTA TGG TTC AAA AGA ATA TTA CAT	Sense

4. Discussion

Generally intracellular uptake of ODN nanoparticles occur via an endocytic–phagocytic process (Fattal et al., 1998). However erythrocyte membrane of infected red blood cells does not show any endocytic properties (Haldar and Uyetake, 1992; Pouvelle et al., 1994). But it is widely recognized that the intracellular malaria parasite induces in the host red blood cell membrane new permeation pathways that are absent from the membrane of the uninfected erythrocytes (Go et al., 2004). Macromolecules like dextrans, protein A and IgG2a antibody were shown to gain access to the parasite through permeation pathways induced by the malaria parasite (Pouvelle, 1991). Using a variety of fluorescent latex spheres, Goodyer et al. determined that macromolecules up to 50–80 nm in diameter access intracellular parasites (Goodyer et al., 1997). In this regard, we developed ODN-chitosan nanoparticles with mean diameters of ∼55 nm. To evaluate the impact of charge, nanoparticles with negative as well as with positive surface charge have been designed, depending on the mass ratio. On the surfaces of the 1:1 mass ratio nanoparticles an excess of ODNs are bound by electrostatic interactions, whereas particles with 2:1 mass ratio an excess of non-neutralised chitosan produced positive charged surfaces.

Stability of nanoparticles under physiological conditions is an important factor influencing the release profile of incorporated ODNs. More easily dissociated complexes mediate a faster onset of action. Köping-Höggard et al. (2004) reported that higher gene expression in vitro as well as in vivo is achieved with less stable chitosan-plasmid DNA complexes (Köping-Höggard et al., 2004). As *P. falciparum* develops from ring to mature trophozoite and schizont stages within 36 h, and antisense ODNs against malarial topoisomerase II were expected to arrest the maturation of throphozoite form (Noonpakdee et al., 2003), thus antisense nanoparticles with a rapid onset of action are required. This notion was supported by the results from this study demonstrating higher inhibition was achieved with nanoparticles displaying a faster release profile compared to more stable chitosan-complexes. However, complexation of ODNs with higher amounts of chitosan leading to more stable particles did not prevent inhibition of parasites. Even after 48 h particles with the 2:1 mass ratio remained stable at pH 7.4 and under physiological salt conditions. However, chitosan has been shown to be degraded in vitro as well as in vivo by enzymes, such as lysozyme and chitosanase, into oligomers (Huang et al., 2004), which assures on the one hand its biocompatibility and on the other hand accelerates drug release from chitosan nanoparticles. PfCHT1, a chitinase gene, has been identified in *P. falciparum* genome (Tsai et al., 2001), but no report is currently available indicating if chitinase is expressed during parasite blood stages. PfCHT1 is reported to be essential for intracellular trafficking and secretion and to be necessary for ookinetes to invade the mosquito midgut (Tsai et al., 2001).

In this study nanoparticles clearly demonstrate a more pronounced sequence specific antisense effect as compared to free ODNs. This could be due to the sustained release of ODNs

from chitosan nanoparticles leading to a relatively lower initial concentration. This contention is in good accordance with previous studies (Noonpakdee et al., 2003; Rapaport et al., 1992; Barker et al., 1998) showing that oligonucleotides inhibit cellular gene expression in a sequence specific manner at low concentrations. At high concentrations (1 μM and more) both sense and antisense ODNs inhibit growth of parasites in a non-specific manner by the polyanionic properties of oligonucleotides which interfere with the merozoite invasion into red blood cells (Noonpakdee et al., 2003; Barker et al., 1998) in a manner similar to that observed with dextran sulphate (Dalton et al., 1991; Kanagaratnam et al., 1998). An additional explanation for the high percent of inhibition induced by sense nanoparticles might be partly attributed to induced perturbations in the intracellular metabolic activity caused by the complexes (Lambert et al., 1998). In the present study an inhibitory effect of free chitosan could be ruled out, however its intracellular uptake might be different than nanoparticles uptake. Even if non-toxic, some complexes, irrespective of their DNA content, are able to modify intracellular signalisation pathways (Filion and Phillips, 1997). However, antisense-nanoparticles presented in this study clearly demonstrate a significant higher inhibition than in comparison with sense-nanoparticles.

Furthermore nanoparticles demonstrated effective protection of oligonucleotides against nuclease degradation that is a major requirement for in vivo use of antisense technology. Another requirement for in vivo use of nanoparticles against malaria is the proof that they do not harm red blood cells. The erythrocyte membrane contains anionic glycoproteins which can interact with protonated amino groups of chitosan. This process induces membrane curvature, leading to rupture and haemoglobin release (Carreno-Gomez and Duncan, 1997). Nanoparticles with positive surface charge showed a higher membrane damaging effect than compared to negatively charged particles. However, in the presence of plasma, haemoglobin release was markedly reduced. The lower membrane damaging effect in the presence of plasma might be explained by absorption of negatively charged plasma proteins on the surface of charged particles, and this shielding effect of plasma suggests that these chitosan nanoparticles may not harm erythrocytes under in vivo conditions. As far as we know this study is the first dealing with nanotechnology against malaria, demonstrating the susceptibility of human malaria parasite, *P. falciparum*, to antisense nanoparticles.

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Inhibition of *Plasmodium falciparum* proliferation *in vitro* by double stranded RNA directed against malaria histone deacetylase

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Inhibition of *Plasmodium falciparum* proliferation *in vitro* by double stranded RNA directed against malaria histone deacetylase

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Abstract

Acetylation and deacetylation of histones play important roles in transcription regulation, cell cycle progression and development events. The steady state status of histone acetylation is controlled by a dynamic equilibrium between competing histone acetylase and deacetylase (HDAC). We have used long *Pf*HDAC-1 double-stranded (ds)RNA to interfere with its cognate mRNA expression and determined the effect on malaria parasite growth and development. Chloroquine- and pyrimethamine-resistant *Plasmodium falciparum* K1 strain was exposed to 1-25 µg of dsRNA/ml of culture for 48 h and growth was determined by [^{3}H]-hypoxanthine incorporation and microscopic examination. Parasite culture treated with 10 µg/ml *pf*HDAC-1 dsRNA exhibited 47% growth inhibition when compared with either untreated control or culture treated with an unrelated dsRNA. *Pf*HDAC-1 dsRNA specifically blocked maturation of trophozoite to schizont stages and decreased *Pf*HDAC-1 transcript 44% in treated trophozoites. These results indicate the potential of HDAC-1 as a target for development of novel antimalarials.

Key words: *Plasmodium falciparum;* Malaria; Double-stranded RNA; Histone deacetylase; *Pf*HDAC-1

Introduction

Malaria is one of the most widespread of human parasitic diseases affecting annually approximately 300 million people worldwide and about 1.5-2.7 million infected individuals, mostly children and pregnant women, die every year from this disease [1,2]. Global efforts to eradicate malaria have failed due to the fact that the most virulent malarial parasite, *Plasmodium falciparum*, has become widely resistant to nearly all currently employed antimalarials and an effective vaccine has not materialized [2,3]. Thus there is an urgent need to develop new classes of antimalarial compounds to combat this serious health problem.

Histones of *P. falciparum* have recently been studied as new targets for blood stage antimalarial drug development [4,5]. Histones are nuclear core proteins, which are essential in packaging DNA into chromosomes. They are highly conserved across phyla, and may play a role in the pathology of malaria [5]. *P. falciparum* H2A, H2B and H3 histones show close homology with histones from other organism [8]. Post-translational acetylation and deacetylation modifications of histones play important roles in transcriptional regulation, cell cycle progression and development events in eukaryotes. The acetylation state of histones is controlled by a dynamic equilibrium between two family of enzymes, histone acetyltransferase and histone deacetylase (HDAC) [5,6]. HDACs can be classified into three categories: class I (HDAC 1, 2, 3 and 8), class II (HDAC 4, 5, 6, 7, 9 and 10) and class III (HDAC sirtulins). Class I and II HDACs have zinc-dependent mechanisms, but those of class III require NAD^+ for their activity [7]. Two histone deacetylase homologues from *P. falciparum, Pf*HDAC-1

and *Pf*Sir2, have been cloned and sequenced [9,10]. *Pf*HDAC-1 nucleotide sequence reveals an open reading frame of 1347 nucleotides encoding a protein of 449 amino acids with the molecular weight of 51.4 kDa. The gene is present as a single copy, shows significant homology to yeast, human and other eukaryotic HDACs and is predominantly expressed in asexual blood stages and also in gametocytes [9].

Histone deacetylase inhibitors, mostly of class I and II enzymes, have been synthesized and shown to have potent and specific anticancer activities, and some are currently undergoing clinical trials [11]. Apicidin, a cyclic tetrapeptide isolated from *Fusarium* spp., inhibits growth of malaria parasites by inhibiting HDAC[4,11]. Other HDAC inhibitors, including SAHA, trichostatin and hydroxamate derivatives, have also been shown to exhibit antimalarial effects [12-14]. Thus, the inhibition of plasmodial HDACs could be a possible approach for the development of novel and selective antimalarial drugs [4,14].

RNA interference (RNAi) has emerged as a powerful method for studying gene function in both unicellular and multicellular organisms and has been applied to the study of functions of many genes associated with human disease, in particular those associated with oncogenesis and infectious diseases [15]. RNAi has also been applied to protozoan parasites, including *Trypanosoma brucei* and *P. falciparum* [16-21]. In the latter RNAi was employed to transiently silence genes of cysteine proteases, transcription factor PfMby1, and bifunctional glucose 6-phosphate dehydrogenase-6-phosphogluconolactonase. In this study, we used a long double-stranded RNA (dsRNA) of *pf*HDAC-1 to interfere with expression of the cognate mRNA, confirming the key role of HDAC-1 in intra-erythrocytic stage of infection.

105

106 **Materials and methods**

107

108 *Parasite culture*. *P. falciparum* K1 strain (chloroquine- and pyrimethamine-
109 resistant) isolated in 1979 from an infected individual in Kanchanaburi province,
110 Thailand was maintained in human erythrocytes in RPMI 1640 medium supplemented
111 with 10% human serum, 25 mM Hepes and 32 mM $NaHCO_3$ under continuous *in vitro*
112 culture using the candle-jar method of Trager and Jensen [22].

113

114 *dsRNA preparation.* Two DNA fragments coding for 349 nt of *pf*HDAC-1 gene
115 (GenBank accession number AF091326) were PCR amplified from *P. falciparum*
116 genomic DNA and used as templates for synthesis of sense (sRNA) and antisense RNA
117 (asRNA) strands. Two pairs of oligonucleotide primers with T7 RNA binding sites used
118 were 5'T7-TCCTATGAAGCCTCAACG and 5'AGCATGATGCAATCCTCC for
119 synthesis of sRNA, and 5'T7-AGCATGATGCAATCCTCC and 5'
120 TCCTATGAAGCCTCAACG for asRNA. PCR amplicons were purified and subjected
121 to sequence analysis using ABI Prism Dye Terminator Cycle sequencing Ready
122 Reaction kit in an ABI Prism 377 DNA sequencer. sRNA and asRNA were then
123 generated using T7 RiboMax Express Large Scale RNA Production System (Promega).
124 Double strand (ds)RNA was prepared by mixing equal amounts of sRNA and asRNA,
125 then heating at 75 °C for 5 minutes and annealing by slow cooling to room temperature
126 over several hours. DsRNA was analyzed by electrophoresis in 1.5 % agarose gel.
127 Unrelated dsRNA (GFP) was generated by using recombinant plasmid expressing stem
128 loop GFP RNA as previously described [23].

129

130 *In vitro assessment of antiplasmodial activity.* Growth of parasites was
131 synchronized to the ring stage by sorbitol treatment [24]. A 200 µl aliquot of 1.5% cell
132 suspension with 1-3% parasitemia was pre-exposed to 25 µl of medium containing
133 various concentrations of dsRNAs, ranging from 1 to 25 µg/ml (4-100 nM), 10 nM
134 trichostatin A (Sigma) or serum-free culture medium. Parasite growth was monitored
135 by measuring incorporation of [^{3}H]-hypoxanthine and by microscopic examination of
136 Giemsa-stained thin blood smears as previously described [25]. Parasite stages were
137 determined by microscopic examination of 5000 red cells under oil immersion. Results
138 were expressed as percent inhibition of parasite growth compared to control receiving
139 medium alone. All values presented are the averages of three experiments conducted in
140 triplicate.
141
142 *RT-PCR assay*. For RNA extraction, 25 µg/ml dsRNA were added to highly
143 synchronized ring stage parasites (5% parasitemia, 4% hematocrit) for 24 h. Control
144 and GFP dsRNA-treated cultures were carried out at the same time. Infected cells were
145 washed with cold RNase-free buffer and centrifuged for 10 min at 600xg at 4 ^{o}C.
146 Parasites were liberated from red cells by incubating with 5 volumes of 0.15% (w/v)
147 saponin in phosphate-buffered saline (PBS) at 37 ^{o}C for 10 min and then sedimenting at
148 5000x*g* at 4 ^{o}C for 10 min. Intact parasites were washed at least three times with
149 RNase-free PBS.
150
151 Total RNA was extracted from 3 ml of dsRNA-treated culture using TRI reagent
152 (Molecular Research Center). One µg of RNA was treated with RNase-free DNase I
153 (Qiagen) and purified using RNeasy column and Qiagen clean-up procedure to remove

DNA contamination. Synthesis of cDNA was performed using random hexamers and ImProm-II reverse transcriptase system (Promega). PCR was carried out with 2-4 µl of cDNA sample (or negative control) in the presence of 0.5 µM primers (18S RNA forward (5′ CATTCG TATTCAGATGTCAGAGGTG) and reverse primers (5′ CGTTCGTTA TCGGAATTAACCAGAC) or *pf*HDAC-1 forward (5′ CCAGATGTGTAGAACACG) and reverse primers (5′ ACACCTGGAGCATGTTCTATGTGTC)), 200 µM dNTP and two units of *Taq* polymerase (New England Biolabs). After denaturation at 94 °C for two minutes, 30 cycles with annealing at 52 °C, elongation at 72 °C and denaturation at 94 °C were performed to amplify the *pf*HDAC-1 fragments. Eighteen cycles of the same amplification profile were used to amplify 18S RNA cDNA fragment. Under these thermocycling conditions, the magnitude of the signals was proportional to RNA concentration. Fragments of expected lengths (482 and 298 bp for 18S RNA and *pf*HDAC-1 respectively) were separated by 1.5% agarose gel-electrophoresis and relative amounts of ethidium bromide-stained amplicons were determined by densitometry.

Results and discussion

Inhibition of parasite growth by pfHDAC-1 dsRNA

In order to demonstrate antiplasmodial effect of *Pf*HDAC-1 dsRNA, growth of ring stage *P. falciparum* K1 in culture was measured in the presence of a 346 bp dsRNA targeting the coding region of parasite histone deacetylase gene or an unrelated GFP

dsRNA of similar size (~ 0.4 kb) (negative control was buffer alone). *Pf*HDAC-1 dsRNA significantly reduced parasite growth (~ 47% with 10 µg/ml), compared with GFP dsRNA (12% with 10 µg/ml), suggesting a sequence-specific inhibition (Figure 1). Inhibition of parasite growth by *pf*HDAC-1 dsRNA was dose dependent in the range of 1-10 µg/ml, but remained at 50% at higher concentrations. This may reflect limiting ability of infected red blood cells to uptake dsRNA and/or limiting amounts of processing enzymes involved in RNA interference mechanism. We have previously observed this phenomenon when antisense oligonucleotides against malarial topoisomerase II were employed [25]. The small inhibitory effect of GFP dsRNA might be due to a sequence-independent effect [23]. The known HDAC inhibitor, trichostatin A, exerted 65% inhibition at 10 nM (Figure 1), and completely inhibited parasite growth at 20 nM (data not shown).

Malarial growth inhibition of approximately 40-60% by dsRNAs has been reported [17-21]. The amounts of dsRNA used in these studies were in the range of 1-50 µg/ml of parasite culture. It has been shown that electroporation of 1 µg/ml dsRNA directed against gene of dihydroorotate dehydrogenase, an enzyme essential for the pyrimidine biosynthesis of *P. falciparum*, inhibits parasite growth up to 60%, but no inhibition was observed without electroporation [17]. However, other studies have shown that adding dsRNAs against *P. falciparum* cysteine protease mRNA or *P. falciparum* transcription factor *PfMyb1* directly to the medium at 25 µg/ml of culture could inhibit growth of 30% or 48% respectively [18,19]. Electroporation might enhance the delivery of dsRNA to the parasites through at least three membrane layers: red blood cell membrane, parasitophorous vacuolar membrane, and parasite plasma membrane. However, parasitized erythrocytes permit the entry of oligonucleotides

when adding directly to the medium while uninfected cells can not, and approximately 0.1-0.15 % of dsRNA in the medium are taken up by parasites [18,26,27].

To further explore the possible step at which *pf*HDAC-1 dsRNA inhibits development of blood stage parasite, assays were performed using 10µg/ml *pf*HDAC-1 dsRNA added to ring stage parasites. Blood smears were performed every six hours and the stages of parasites were counted. At 24 hours, the numbers of trophozoites in *pf*HDAC-1 dsRNA-treated cultures were comparable to those of controls (Figure 2). However, after 36 hours of exposure to *pf*HDAC-1 dsRNA, schizont stage parasites were 45% of control, a level comparable with that obtained from parasite growth assay, indicating that dsRNA specifically affected parasite maturation from trophozoite to schizont stages.

Decreased expression of pfHDAC-1 mRNA by pfHDAC-1 dsRNA

In order to determine whether inhibition of parasite growth by *Pf*HDAC-1 dsRNA might have resulted from interference of its cognate mRNA, expression of the *pf*HDAC-1 transcript was analyzed after 24 h treatment at the trophozoite stage. Expression of *pf*HDAC-1 is low at the ring stage and rises to a high level in both trophozoite and schizont stages [9]. RT-PCR evaluation of *pf*HDAC-1 transcript levels in parasites treated with *pf*HDAC-1, with GFP dsRNA, or left untreated demonstrated that *pf*HDAC-1 transcript was decreased (44%) in *pf*HDAC-1 dsRNA-treated parasites compared with untreated control or with GFP dsRNA (Figure 3). Data were normalized to 18S RNA. Thus, it is likely that *pf*HDAC-1 dsRNA added exogenously to culture during the period of optimal expression of parasite HDAC-1 reduces its cognate mRNA

level and thereby affecting parasite maturation. This observation is consistent with studies previously reporting that long dsRNAs against *P. falciparum* genes encoding key enzymes (viz. cysteine proteases, dihydroorotate dehydrogenase, helicase) decrease their gene transcripts and reduce growth of parasites in culture [18-21].

The mechanism by which gene expression is modified by dsRNA in *Plasmodium* is still not clearly defined. As typical RNAi-associated genes have not yet been identified in *P. falciparum* genome, it has been suggested that the inhibitory effect of dsRNA might be due to an antisense effect rather than through a classical RNA interference mechanism [17,19,25,28] Interestingly, many antisense transcripts have been found during malaria parasite intra-erythrocytic cycle and such molecules have been suggested to be regulatory elements in gene transcription [29]. It is possible that the machinery required to process these antisense transcripts is present in *P. falciparum* and might be responsible for the resulting dsRNA. Another possibility is that the essential factors in RNAi processes (dicer and RISC) are transported into the intracellular parasite from human host cell [28]. Data presented here showing the susceptibility of *P. falciparum* to dsRNA against a gene of histone deacetylase, a key enzyme involved in cell proliferation and differentiation, validates this target in the development of novel antimalarial agents.

Acknowledgements

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Figure legends

Figure 1. *Pf*HDAC-1 dsRNA inhibition of *P. falciparum* K1 growth in culture. *Pf*HDAC-1 dsRNA or unrelated GFP dsRNA was added to parasite culture at ring stage. After 48 h of incubation, parasitemia was determined from [^{3}H]-hypoxanthine incorporation and shown as mean ± SEM of 3 experiments conducted in triplicate. 1, medium alone; 2, 1 µg/ml; 3, 3 µg/ml; 4, 5 µg/ml; 5, 10 µg/ml; 6, 25 µg/ml; 7, 10 nM trichostatin A.

Figure 2. Stage specificity of *Pf*HDAC-1 dsRNA inhibition of *P. falciparum* K1 growth in culture. *Pf*HDAC-1 dsRNA was added to ring stage parasites for 24 and 36 hours. Parasitemia of trophozoite (1 and 2) and schizont stages (3 and 4) were determined microscopically and expressed as mean percent ± SEM of 3 experiments conducted in triplicate. 1 and 3, medium alone; 2 and 4, *Pf*HDAC-1 dsRNA treated cultures.

Figure 3. RT-PCR analysis of *Pf*HDAC-1 mRNA expression. *Pf*HDAC-1 dsRNA or unrelated GFP dsRNA was added to parasite culture at ring stage for 24 hours. Amplicons of *Pf*HDAC-1 and 18 S ribosomal RNA, obtained by RT-PCR, were examined in ethidium bromide-stained agarose gel. Lanes 1 and 4, untreated culture; lanes 2 and 5, treated with GFP dsRNA; lanes 3 and 6, treated with *Pf*HDAC-1 dsRNA; lane M, molecular size markers.

443 Figure 1

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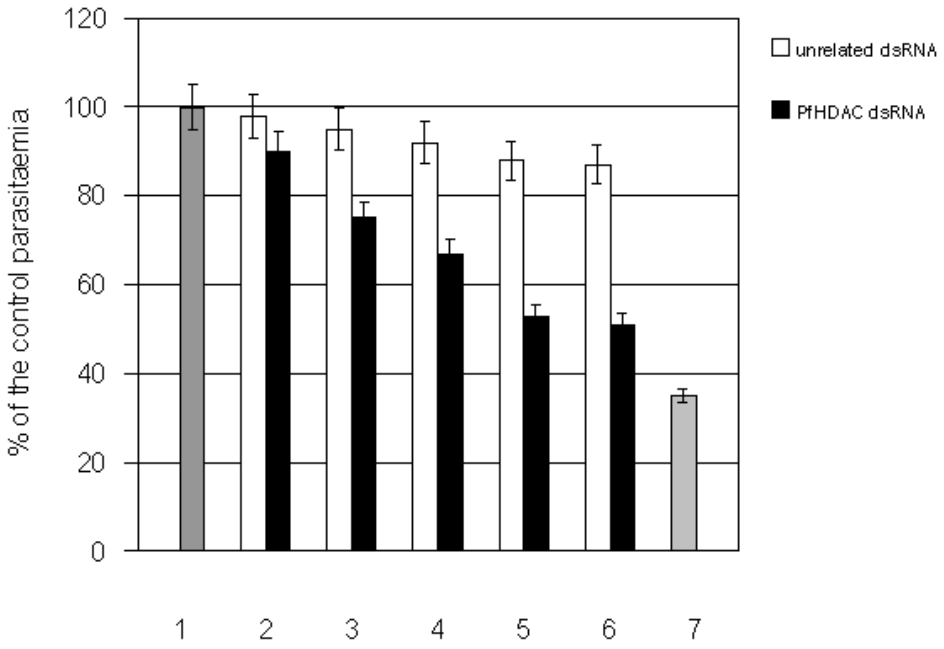

445

446 Figure 2

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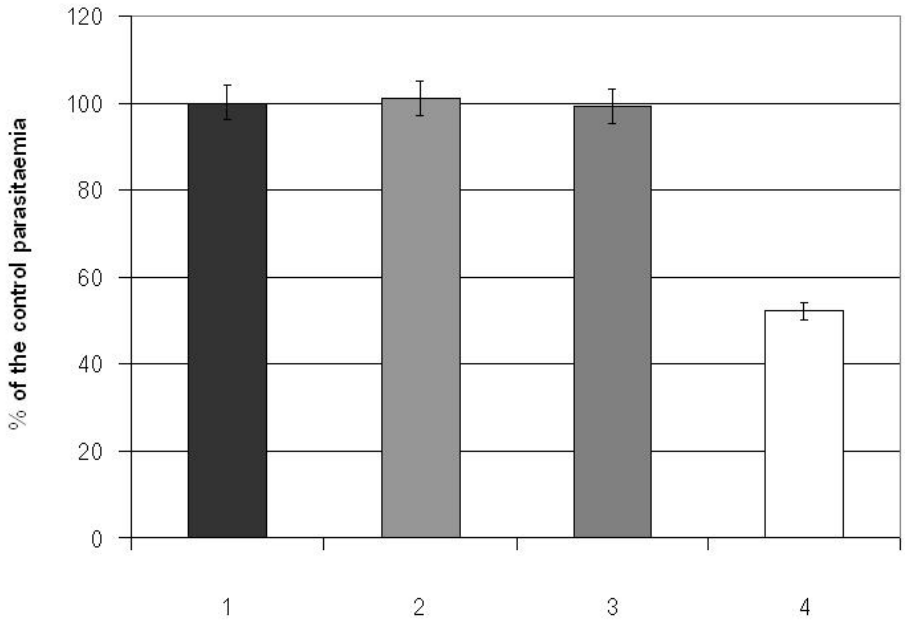

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449 Figure 3

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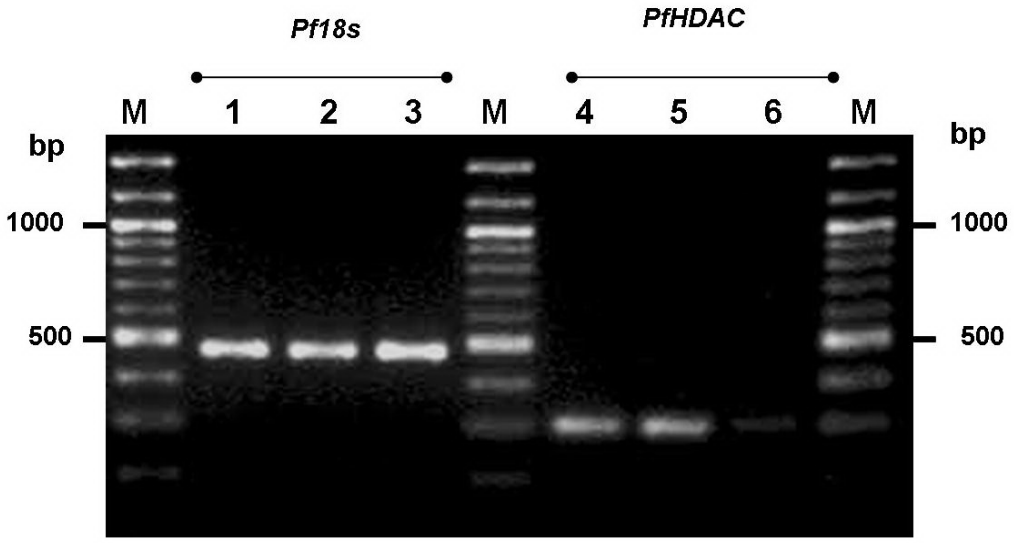

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Inhibition of *P. falciparum* proliferation by antisense and antisense nanoparticles against malarial topoisomerase II

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The development of new effective antimalarial agents is urgently needed due to the ineffectiveness of current drug regimens on the most virulent human malarial parasite Plasmodium falciparum. Antisense oligodeoxynecleotides (ODNs) have shown promise as chemotherapeutic agents. Exogenous delivery of phosphorothioate AS ODNs against different regions of P. falciparum topoisomerase II gene to P. falciparum K1 strain between 0.01-0.5 uM significantly inhibited parasite growth compared with sense sequence control suggesting sequence-specific inhibition by fluorescence-activated cell sorter assay or by microscopic assay. This inhibition was shown to occur during maturation stages, to improve stability and to increase intracellular penetration, ODNs were complexed with the biodegradable polymer chitosan to form solid nanoparticles with an initial diameter of 55 nm. AS nanoparticles showed stronger inhibition of parasite srowth compared with free AS ODNs. Therse results should prove useful in future designs of novel antimalarial agents and use of nanoparticles to delivery ODNs.

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Double stranded RNA mediated gene silencing of histone deacetylase of P. falciparum

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Abstract

The histones of *P. falciparum* have been proposed as a potential new target for antimalarial compounds. They are present in abundant and may play important roles during parasites development stages and proliferation. Histone deacetylases are enzymes involving in acetylation/deacetylation process of histone and control of gene regulation. We used long *pfHDAC* double-stranded RNA(dsRNA) to interfere with the cognate messenger expression. Chloroquine-and pyrimethanmine-resistant *P. falciparum* K1 strain was exposed to *pfHDAC* dsRNA for 48 h and growth was determined by hypoxanthine incorporation assay. Exogeneously delivery of *pfHDAC* dsRNA between 1- 200 nM significantly inhibited parasite growth up to 47 % as compared with either untreated cultures or cultures treated with unrelated dsRNA(gfp) which had some inhibitory effect (15%). In addition, the decrease in parasite growth correlated with the decrease in levels of *pfHDAC* mRNA.

Keywords: double stranded RNA, Gene silencing, Histone deacetylase , P. falciparum

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Inhibition Of *P.falciparum* Proliferation *in vitro* by Antisense Nanoparticles against malarial Topoisomerase II

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New effective antimalarial agents are urgently needed due to increasing drug resistance of *Plasmodium falciparum*. Phosphorothioate antisense oligodeoxynucleotides (ODNs) silencing of malarial topoisomerase II gene have shown to possess promising features as anti malarial agents. In order to improve stability and to increase intracellular penetration, ODNs were complexed with the biodegradable polymer chitosan to form solid nanoparticles with an initial diameter of approximately 55 nm The particle zetapotential depended on the chitosan/ODN mass ratio. Nanoparticles with mass ratio of 2:1 displayed a positive surface charge (+15 mV) whereas particles with 1:1 mass ratio were negatively charged (-20 mV). *P. falciparum* K1 strain was exposed to the chitosan/ODN-nanoparticles for 48 h in order to examine the effects of chitosan/antisense (AS) and chitosan/sense (S) oligodeoxynucleotide nanoparticles on malaria parasite growth. Both negatively and positively charged antisense nanoparticles as well as free antisense ODNs (in a final concentration of 0.5 µM) showed sequence specific inhibition compared with sense sequence controls. However, nanoparticles were much more sequence specific in their antisense effect than free ODNs. Nanoparticles with negative surface charge exhibited a significantly stronger inhibitory effect (87% inhibition) on the parasite growth in comparison to the positive ones (approx. 74% inhibition) or free ODNs (aprrox. 68% inhibition). The ODN antisense nanoparticle with the bigger size of about 70 nm was also tested with 1:1 mass ratio with negatively charged. The result was similar to that of ODN nanoparticles with smaller size. This is the first study demonstrating the susceptibility of P.falciparum to antisense nanoparticles. The results should prove to be useful in future designs of novel antimalarial agents and use of nanoparticles to delivery ODNs.

Key word: Antisense ; Nanoparticles; topoisomeraseII, *P.falciparum* , Malaria

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Double-stranded RNA mediated gene silencing of topoisomerase II of *Plasmodium falciparum*.

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Malaria remains a public health problem. The development of new effective antimalarial agents is urgently needed. RNA interference (RNAi) technology is a tool for studying gene function by interrupting gene expression. We used double-stranded RNA (dsRNA) encoding a segment of the gene encoding *P. falciparum* topoisomerase II to demonstrate the RNAi effect in *P. falciparum*. The DNA fragment approximately 120 and 400 bp in length coding for *P. falciparum* topoisomerase II gene was amplified by PCR using parasite genomic DNA. The PCR product was cloned into pET17b vector to generate dsRNA in *Escherichia coli* HT115. We have tested the ability of topoisomerase II dsRNA to inhibit the *in vitro* growth of chloroquine-and pyrimethamine-resistant *P. falciparum* K1 strain by [3H] hypoxanthine incorporation assay. The growth inhibition was dose dependent in the range of 10-120 nM and was saturated between 120-300 nM. There was no growth inhibition in the untreated culture and slightly inhibition with the unrelated dsRNA-GFP. The correlation of growth inhibition and the mRNA level of *P. falciparum* topoisomerase II transcribed are now under investigated by RT-PCR technique. These results should prove that dsRNA of topoisomerase II gives growth parasite inhibition and pave the way to develop a cure of malaria.

Key word: double stranded RNA; RNAi ; topoisomeraseII, *P.falciparum* , Malaria

Poster presentation: Joint International Tropical Medicine Meeting 2007 “Health Security in the Tropics” 29-30 November 2007, Imperial Queen’s Park Hotel, Bangkok, Thailand

การยับยั้งการแสดงออกของยีน topoisomerase II ใน *Plasmodium falciparum* ด้วย RNA สายคู่

DOUBLE-STRANDED RNA MEDIATED GENE SILENCING OF TOPOISOMERASE II OF *Plasmodium falciparum*

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บทคัดย่อ: มาลาเรียเป็นปัญหาด้านสุขภาพที่สำคัญ การพัฒนายารักษาโรคมาลาเรีย จึงเป็นสิ่งจำเป็น เทคโนโลยี RNA interference (RNAi) เป็นเครื่องมือที่มีประสิทธิภาพในการศึกษาหน้าที่ของยีน โดยไปขัดขวางการแสดงออกของยีน การทดลองนี้ ได้ใช้ RNA สายคู่ที่มีลำดับเบสจำเพาะกับบางส่วนของยีน topoisomerase II ของเชื้อ *P. falciparum* เพื่อพิสูจน์ว่า RNAi มีผลต่อ *P. falciparum* ชิ้นส่วนของยีน topoisomerase II ขนาด 120 และ 400 เบส ถูกเพิ่มจำนวนโดยเทคนิค PCR โดยมี genomic DNA ของ *P. falciparum* เป็นต้นแบบ DNA ที่ได้จากปฏิกิริยา PCR ถูก clone เข้า pET17b vector เพื่อสร้าง RNA สายคู่ใน *Escherichia coli* HT115 โดยทดสอบความสามารถการยับยั้งการเจริญเติบโตของ *P. falciparum* สายพันธุ์ K1 (chloroquine-and pyrimethamine-resistant) ด้วย [^{3}H] hypoxanthine incorporation assay พบว่าการยับยั้งการเจริญเติบโตเป็น dose dependent ในช่วง 10-120 nM และคงที่ในช่วง 100-300 nM และไม่พบการยับยั้งใน untreated culture ส่วน dsRNA-GFP ยับยั้งเพียงเล็กน้อย โดยทดสอบความสัมพันธ์ระหว่างการยับยั้งการเจริญเติบโตและการลดลงของ mRNA ของยีน topoisomerase II ด้วยวิธี RT-PCR ซึ่งกำลังดำเนินการทดลอง และจากผลการทดลองที่ได้พบว่า RNA สายคู่ของยีน topoisomerase II สามารถยับยั้งการเจริญเติบโตของ parasiteได้นับว่าเป็นอีกแนวทางหนึ่งที่สามารถพัฒนายารักษาโรคมาลาเรียต่อไป

Abstract: Malaria remains a public health problem. The development of new effective antimalarial agents is urgently needed. RNA interference (RNAi) technology is a tool for studying gene function by interrupting gene expression. We used double-stranded RNA (dsRNA) encoding a segment of the gene encoding *P. falciparum* topoisomerase II to demonstrate the RNAi effect in *P. falciparum*. The DNA fragment approximately 120 and 400 bp in length coding for *P. falciparum* topoisomerase II gene was amplified by PCR using parasite genomic DNA. The PCR product was cloned into pET17b vector to generate dsRNA in *Escherichia coli* HT115. We have tested the ability of topoisomerase II dsRNA to inhibit the *in vitro* growth of chloroquine-and pyrimethamine-resistant *P. falciparum* K1 strain by [^{3}H] hypoxanthine incorporation assay. The growth inhibition was dose dependent in the range of 10-120 nM and was

saturated between 120-300 nM. There was no growth inhibition in the untreated culture and slightly inhibition with the unrelated dsRNA-GFP. The correlation of growth inhibition and the mRNA level of *P. falciparum* topoisomerase II transcribed are now under investigated by RT-PCR technique. These results should prove that dsRNA of topoisomerase II gives growth parasite inhibition and pave the way to develop a cure of malaria.

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