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Effects of Host Hemoglobinopathic Red Blood Cells on the Sensitivity of *Plasmodium falciparum* to Dihydroartemisinin and Piperaquine

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ABSTRACT

The adaptation of *Plasmodium falciparum*-infected hemoglobinopathic red blood cells (RBCs) influences the sensitivity of the parasite to antimalarial drugs which may lead to drug resistance. This study aimed to investigate *in vitro* sensitivity and interaction of *P. falciparum*-infected RBCs with normal hemoglobin and hemoglobinopathic RBCs, to dihydroartemisinin (DHA) and piperaquine (PIP). Additionally, the effect on *Pf*PI3K enzyme activity via PI(3)P production was also investigated. Twenty-five RBC samples were co-cultured with 3D7 (chloroquine-sensitive) and K1 (chloroquine-resistant) *P. falciparum* clones. The samples covered several RBC variants including: normal hemoglobin (HbA), Hemoglobin E trait (HbE trait), *β*-thalassemia trait, *α*-thalassemia trait, or hemoglobin Constant Spring trait (HbCS trait). Parasite sensitivity to DHA and PIP was investigated using the SYBR green method. PI(3)P production was measured by ELISA. This study showed that 3D7 P. falciparum infected-RBCs with HbE trait and HbA were less sensitive to DHA. On the other hand, the parasite-infected *β*-thalassemia trait RBCs were the most sensitive to PIP compared to the others. For K1 *P. falciparum*, a decrease in DHA and PIP sensitivity was found only in the parasite-infected *β*-thalassemia RBCs. No antimalarial drug interaction was observed between DHA and PIP in either of the *P. falciparum* strains except for K1-infected *α*-thalassemia trait RBCs (additive effect). DHA exposure significantly increased PI(3)P expression (>2.5 fold) in 5 3D7 *P. falciparum*-infected samples, in both normal and hemoglobinopathic RBCs. PIP exposure had no influence

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on PI(3)P expression. The results suggest that hemoglobinopathic RBCs slightly affect parasite sensitivity to DHA and PIP and *Pf*PI3K activity via PI(3)P production, which may be related to mutation of the *Pf*Kelch13 gene and results in artemisinins resistance.

Keywords: dihydroartemisinin (DHA); *Plasmodium falciparum*; hemoglobinopathy; piperaquine (PIP); phosphatidylinositol-3-phosphate [PI(3)P]; phosphatidylinositol-3-kinase (PI3K)

1. Introduction

α-, β-Thalassemia, and hemoglobin E are hemoglobin (Hb) polymorphisms commonly found in Southeast Asia, overlapping with malaria endemicity [1]. These Hb variants have been reported to protect humans from malaria infection. A small number of Hb abnormalities in heterozygous carriers, however, do not confer protection against malaria infection [2]. Incomplete Hb production results in Hb debris and induces oxidative stress, which may influence antimalarial treatment response [3]. Accumulation of the antimalarial drug artemisinin at the red blood cell (RBC) membrane compartment (instead of the cytosol) has been reported in some hemoglobinopathic RBCs such as hemoglobin H (HbH), HbH/Hb CS, and β-thalassemia/ hemoglobin E (HbE), which results in reduced antimalarial sensitivity [4].

The long-term exposure of malaria parasite to antimalarial drugs without complete parasite clearance can produce selective drug pressure and the development of parasite drug-resistance. To cope with multidrug-resistant malaria, particularly in Southeast Asia, the World Health Organization (WHO) recommends using artemisinin combination therapies (ACTs) as the first-line treatment for uncomplicated falciparum malaria in all endemic areas. In Thailand, the artesunate-mefloquine combination was initially introduced for malaria control in 1994, but 20 years later, its 42-day clinical efficacy was reduced to 80% [5, 6]. That combination was then replaced by dihydroartemisinin (DHA)-piperaquine (PIP) combination therapy in 2015 [7, 8]. The fix-dose DHA-PIP combination facilitates patient compliance with medication and reduces the risk of drug resistance. A high clinical efficacy and safety profile has been well documented in various malaria endemic areas, including artemisinin resistant areas [9-13]. The antimarial activity of DHA is related to the production of free radicals from the oxygen molecules that react with ferrous iron which are toxic to the malaria parasite [14]. The resistance of *P. falciparum* to artemisinin drugs, including DHA, has been reported in Southeast Asian countries, and is associated with the mutation of the *P. falciparum* Kelch13 (*Pf*Kelch13) gene [15]. DHA inhibits *P. falciparum* phosphatidyl-inositol 3-kinase (*Pf*PI3K) enzyme activity, resulting in low phosphatidylinositol 3-phosphate [PI(3)P] production. The *Pf*Kelch13 gene mutation decreases binding affinity to the PI3K enzyme, resulting in the reduction of PI3K degradation by polyubiquitination and an increase in PI(3)P production [16]. The combination partner PIP is a chloroquine (CQ) derivative with a relatively long half-life of about 1 month. It exhibits antimalarial activity against both CQ-sensitive and CQ-resistant *P. falciparum* [17]. The mode of action of PIP remains unclear but is thought to be similar to chloroquine, i.e., interference with heme polymerization. The mechanism of resistance, however, appears to be different from CQ [17, 18].

DHA-PIP combination therapy was introduced in Thailand after artemisinin resistance had been reported in Southeast Asia. This may result in the induction of resistant pressure on PIP and treatment failure following DHA-PIP combination therapy. Therefore, investigation of the possible factors resulting in variability in treatment response would help to predict the

treatment efficacy of DHA-PIP in Thailand. The present study aimed to investigate *in vitro* sensitivity to DHA and PIP, including antimalarial interaction of *P. falciparum*-infected RBCs with normal hemoglobin (HbA) and hemoglobinopathic RBCs. The association between *Pf*PI3K enzyme activity and PI(3)P production was also determined.

2. Materials and Methods

2.1 Chemicals and reagents

RPMI, HEPES, gentamicin, and albumax II were supplied by Invitrogen (Carlsbad, CA, United States). CQ, DHA, piperaquine tetraphosphate tetrahydrate (PIP), SYBR Green I, saponin, and trichloroacetic acid (TCA) were purchased from Sigma Aldrich Inc. (St. Louis, MO, USA). Ethanol, methanol, and chloroform were purchased from RCI Labscan Co. Ltd. (Bangkok, Thailand). Ethylenediaminetetra- acetic acid (EDTA) was purchased from Affymetrix/USB (Santa Clara, CA, USA). PBS was purchased from VWR (Radnor, PA, USA). PI(3)P Mass ELISA (K-3300) was purchased from Echelon Biosciences (Lake City, UT, USA).

2.2 Blood samples

The Ethics Committee for Research in Human Subjects of Thammasat University approved the study protocol (project no. 090/ 2559). Venous blood samples (18 ml each) were collected (into vacuum EDTA tubes) from 25 healthy Thai subjects (verified by laboratory and clinical investigation i.e.; measurements of complete blood count (CBC), blood pressure, pulse rate, and body temperature, as well as information on congenital disease, illness, and medication used within the last 2 weeks. Written informed consent was obtained from each subject before blood collection.

2.3 RBC classification

RBC samples ($n = 25$) were classified into 3 groups based on capillary electrophoresis (CE) Hb typing and polymerase chain reaction (PCR) : (i) normal hemoglobin (HbA) RBCs ($n = 10$), (ii) β-globin polymorphism RBCs ($n = 12$), consisting of 10 samples with hemoglobin E trait (HbE trait) and 2 samples with β-thalassemia trait, and finally (iii) α- globin polymorphism RBCs $(n = 3)$, consisting of 2 samples with α- thalassemia, including 1 α- thalassemia-1 trait (SEA deletion) and 1 α- thalassemia-2 trait (3.7 kb+ 4.2 kb deletion) and 1 sample with hemoglobin Constant Spring trait (HbCS trait).

2.4 Parasite cultivation

3D7 (CQ- sensitive) and K1 (CQ-resistant) *Plasmodium falciparum* clones were continuously cultured in 5% RBCs in complete RPMI-1640 medium containing 0.2% $NaHCO_3$, 10% serum, 25 mM HEPES (pH 7.4), and 15 mg/ ml gentamicin in 5% CO_2, 5% O_2 and 90% N_2 atmosphere at 37 °C [19]. Parasites were maintained at 5% parasitemia and subcultured every 48 hours. Ring stage parasites were obtained by synchronization with 5% sorbitol.

2.5 Investigation of parasite invasion

Ring stage 3D7 *P. falciparum* (5% parasitemia) was mixed with either HbA or hemoglobinopathic RBCs at a ratio of 1:10 (infected RBCs: uninfected RBCs) to obtain a final cell suspension with 0.5% parasitemia. The infected- cell suspension with 1% hematocrit was prepared by further diluting the cell suspension with complete media. Then a 100 µl aliquot was added into a 96-well plate. The plate was incubated under 5% CO_2, 5% O_2, and 90% N_2 atmosphere at 37 °C for 48, 96, and 144 hours. Parasites were then harvested, and a thin film was prepared. Parasite density and morphology were examined under a microscope with 100x objective lense.

2.6 Assessment of *in vitro* sensitivity

Ring stage 3D7 *P. falciparum* were cultured with HbA RBCs as well as hemoglobinopathic RBCs at a ratio of 1:20 (infected RBCs: uninfected RBCs) for at least 48 hours. The infected cell suspension was prepared (2% parasitemia and 1% hematocrit) for the *in vitro* sensitivity test according to the methods of Rieckmann and Bennette [20, 21]. Stock solutions of CQ and DHA were prepared (10 mM in 50% ethanol). A stock solution of PIP was prepared as well (10 mM in 0.5% lactic acid) [22]. Working solutions of all reference compounds were prepared by further diluting the stock solutions with complete media. Parasite suspensions (100 µl) containing either DHA (0.16-20 nM), PIP (2.34-300 nM), or CQ (20 nM) were incubated at 37 °C for 48 hours to assess the sensitivity of the 3D7 and K1 parasites to each drug. To evaluate the sensitivity of DHA and PIP in combination, DHA (40 nM) and PIP (600 nM) were initially mixed then further diluted to prepare mixtures at ratios of 3:7, 5:5, and 7:3, as well as PIP alone and DHA alone. The microtiter plate was incubated at 37 °C for 48 hours. Parasite DNA content was stained by SYBR green I diluted in lysis buffer, and fluorescence intensity was measured using a microplate reader (Verioskan flash™, Thermo, MA, USA) at an excitation and emission wavelengths of 485 and 530 nm, respectively [20]. Each experiment was repeated in triplicate.

IC_{50} values (drug concentration that inhibits parasite growth by 50%), an indicator of parasite drug-sensitivity, were determined by analyzing the log-dose-response curve (Calcusyn™ version 1.1, BioSoft, Cambridge, UK). The sum FIC_{50} value (DHA IC_{50} from each combination ratio/ DHA IC_{50} of a single drug) + (PIP IC_{50} from each combination ratio/PIP IC_{50} of a single drug) was used as the indicator for the sensitivity of the parasite strains to the DHA-PIP combination. Sum FIC was applied to identified interactions as being synergistic, additive, non- interactive, or antagonistic (FIC < 0.5, 0.5-1.0, 1.0-4.0, and > 4.0, respectively) [22, 23]. Isobologram plots were also prepared with three additional lines, at the 0.5:0.5, 1.0:1.0 and 4.0:4.0 axes to visualize the interactions. ‘Antagonistic effect’ was indicated if the curves lied above the 4.0:4.0 axes.

‘No interaction' was indicated if the curves lied between the 1.0-4.0 axes. ‘Additive effect’ was indicated if the curves lied between the 0.5-1.0 axes. 'Synergistic effect' was indicated if the curves lied below the 0.5 axes [23-25].

2.7 Determination of phosphatidylinositol 3-phosphate [PI(3)P] production

3D7 *P. falciparum* was cultured with RBCs (2% parasitemia and 1% hematocrit) carrying normal or abnormal Hb for at least 48 hours. DHA (4 nM) or PIP (40 nM) in complete media containing albumax (II 0.5%) was exposed to parasite suspension for 6 [16] and 24 hours, respectively. Complete media was used as a negative (untreated) control. The parasite was harvested, and RBCs were lysed using cold saponin (0.1%, w/v). The pellet was washed with 5% trichloroacetic acid (TCA, 5%) in EDTA (1 mM). The lipid component was separated by extraction twice with a mixture of methanol and chloroform (2:1, v/v) at 25 °C for 10 minutes. The pellet was further separated with a mixture of methanol, chloroform, and hydrochloric acid (12 M) (80:40:1, v/v/v) at 25 °C for 25 minutes. The contaminated proteins were separated from the cell supernatant by mixing with chloroform and hydrochloric acid (0.1 N). The organic phase (bottom layer) was collected, dried under nitrogen stream, and kept at -20 °C until use. The concentration of PI(3)P was measured according to the manufacturer’s protocol (K-3300, Echelon Biosciences, Lake City, UT, USA) [16]. Briefly, the mixture of tested samples or PI(3)P standard solution (100 µl each) were added to ELISA plates coated with the first PI(3)P antibody and incubated at 37 °C for 1

hour before removing non- specific compounds by washing with PBS-tween (PBS-T, provided in the kit). The secondary antibody tagged with the detector was incubated with the plate for 1 hour and the excess compounds were removed by washing with PBS-T. The substrate was added to the plate, and the reaction was stopped by adding H_2SO_4 (1 N). The amount of PI(3)P correlated to absorbance reading by a microplate reader at 450 nm. The effects of DHA and PIP on PI(3)P production were determined from the ratio of PI(3)P in the drug- exposed sample and PI(3)P in the untreated sample.

2.8 Data analysis

Quantitative data including CBC parameters, parasite density, IC_{50}, sum FIC_{50}, and PI(3)P concentration are presented as median (95% CI). Quantitative groups were compared using the Mann- Whitney U test. The statistical significance level was set at α = 0.05. The analysis was performed by SPSS version 15.0 (SPSS, Chicago, IL, USA).

3. Results

3.1 Blood sample analysis

The results of all RBC samples, shown in Table 1**,** had similar CBC parameters except mean corpuscular volume (MCV). The MCV of HbA blood samples were significantly higher than those of β- and α- globin polymorphisms. Median Hb (95% CI) levels in HbA, HbE trait, β- thalassemia trait, α- thalassemia trait, and HbCS trait RBCs were 14.3 (12.2-15.0), 12.6 (10.2- 13.6), 11.2 (11.2- 11.3), 11.2 (11.1- 11.3), and 13.9 g/dL, respectively.

The MCV of HbA RBCs was higher [85.3 (77.3-87.8) fL] than HbE [73.7 (64.7- 83. 6) fL)], β- thalassemia trait (65. 4 fL, $n=1$), and α- thalassemia trait [68.0 (67.0- 69. 1) fL] RBCs. The hematocrits of HbA trait RBCs [43. 1 (35. 9- 45. 0)%] and HbCS RBCs (44. 6%, $n=1$) were significantly higher than HbE [38. 2 (32. 3- 41. 2)%], β- thalassemia trait [36. 4 (36. 1- 36. 8)%], and α- thalassemia trait [35. 4 (34. 5- 36. 3)%] RBCs.

Table 1. Blood sample analysis. Data are presented as median (95% CI) values.

Hemoglobin polymorphisms	*n*	Complete blood count (CBC)[a]			
		Red Blood cell, RBCs ($\times 10^6$/uL)	Hemoglobin (g/dL)	Hematocrit (%)	Mean Corpuscular Volume, MCV (fL)[b]
Normal hemoglobin	10	5.0 (4.1-5.3)	14.3 (12.2-15.0)	43.1 (35.9-45.0)	85.3 (77.3-87.8)
Hemoglobin E trait	10	5.0 (4.8-5.8)	12.6 (10.2-13.6)	38.2 (32.3-41.2)	73.7 (64.7-83.6)
β-thalassemia trait	2	5.7 (5.5-6.0)	11.2 (11.2-11.3)	36.4 (36.1-36.8)	65.4[c]
α-thalassemia trait	2	5.2 (5.0-5.4)	11.2 (11.1-11.3)	35.4 (34.5-36.3)	68.0 (67.0-69.1)
Hemoglobin CS trait	1	5.2	13.9	44.6	-

[a] The CBC parameters were not significant different among the RBC polymorphisms as *p*-value >0.05.
[b] fL stands for femtoliter
[c] *n*= 1

3.2 Effects of hemoglobinopathic RBCs on *P. falciparum* invasion

Hemoglobin polymorphisms [HbE trait $(n=7)$, β- thalassemia trait $(n=2)$, α- thalassemia trait $(n=2)$, and HbCS trait $(n=1)$] slightly affected the invasion, growth, and multiplication of 3D7 *P. falciparum,* as shown in Table2. Following 48 hours of incubation (1 multiplication period), parasite density for HbA RBCs $(n=5)$ was higher than HbE trait $(n=7)$, β- thalassemia trait $(n=2)$, α- thalassemia trait

$(n=2)$, and HbCS trait $(n=1)$ RBCs. When extending the incubation period to 96 hours (2 multiplication periods), the parasite density for HbA RBCs was found to be highest [2.99 (2.11-5.397) %] when compared to HbE trait [1.60 (0.575-3.341) %], β-thalassemia trait [0.73 (0.483-0.975) %], α-thalassemia trait [1.67 (0.761-2.576) %], and HbCS trait (1.20 %) RBCs. However, following an incubation period of 144 hours (3 multiplication periods), the parasite density of α-thalassemia trait RBCs [1.92 (0.874-2.961) %] was found to be the highest when compared with the other infected hemoglobinopathic RBCs as well as infected HbA RBCs [1.19 (0.935-4.96) %].

In addition to this, the median (95% CI) invasion of 3D7 *P. falciparum* to RBCs during the 48-, 96-, and 144-hour incubation periods were calculated. The parasite density of HbA, HbE trait, β-thalassemia trait, α-thalassemia trait, and HbCS trait RBCs were 1.19 (1.044-2.991)%, 0.87 (0.868-1.601)%, 0.73 (0.637-0.858) %, 1.67 (0.989-1.917) %, and 1.01 (0.359-1.202) %, respectively. Parasite morphology following the 3 multiplication periods was similar, although relatively slower parasite development was observed in β- and α-thalassemia trait RBCs, and a relatively lower number of merozoites was observed in the infected α-thalassemia trait RBCs.

Table 2. *P. falciparum* invasion during 48-, 96- and 144-hours incubation.

Hemoglobin polymorphisms	n	Parasitemia at H48, H96 and H144 (%)[a,b]			
		H48	H96	H144	Overall 3 periods
Normal hemoglobin	5	1.04 (0.935-1.460)	2.99 (2.11-5.397)	1.19 (0.935-4.96)	1.19 (1.044-2.991)
Hemoglobin E trait	7	0.87 (0.624-1.396)	1.60 (0.575-3.341)	0.87 (0.626-1.396)	0.87 (0.868-1.601)
β-thalassemia trait	2	0.64 (0.551-0.723)	0.73 (0.483-0.975)	0.86 (0.483-0.975)	0.73 (0.637-0.858)
α-thalassemia trait	2	0.99 (0.874-1.105)	1.67 (0.761-2.576)	1.92 (0.874-2.961)	1.67 (0.989-1.917)
Hemoglobin CS trait	1	0.36	1.20	1.01	1.01 (0.359-1.202)

[a] Parasitemia at H0 is 0.5%
[b] The percent invasion during H48, H96 and H144 were not significant different as *p*- value >0.05.

3.3 Effects of hemoglobinopathic RBCs on the sensitivity of *P. falciparum* to dihydroartemisinin and piperaquine

3.3.1 Single drug therapy

The sensitivity of 3D7 and K1 *P. falciparum*-infected RBCs to DHA and PIP were not significantly different between HbA RBCs and hemoglobinopathic RBCs (HbE, α-thalassemia trait, and β-thalassemia trait RBCs) (p>0.05) (Tables 3 and 4).

The 3D7 *P. falciparum* appeared to be less sensitive to DHA [IC_{50}: 3.42 (2.69-4.90) nM)] but more sensitive to PIP [IC_{50}: 35.9 (28.8-43.2) nM)]. In contrast, the K1 *P. falciparum* appeared to be more sensitive to DHA [IC_{50}: 2.45 (1.65-3.40) nM)] but less sensitive to PIP [IC_{50}: 62.3 (50.2-68.5) nM)].

3.3.2 Combination therapy

The interaction between DHA and PIP was evaluated based on sum FIC_{50} values and isobolograms (Tables 3 and 4). An additive effect was found only in K1 infected α-thalassemia trait RBCs [median (95%CI) of sum FIC_{50}=0.883 (0.662-1.000) and all data points located between 0.5-1.0 axes of additive lines]. No interaction was observed in either 3D7 or K1 *P. Falciparum*-infected HbA, HbE trait, and β thalassemia trait RBCs, as well as 3D7 infected α-thalassemia trait RBCs (sum FIC_{50} > 1.0-4.0 and all data points were within 1.0-4.0 axes of the additive lines).

Table 3. Dihydroartemisinin (DHA) and piperaquine (PIP) IC_{50} (nM) in 3D7 *P. falciparum*-infected RBCs with normal hemoglobin or hemoglobinopathic RBCs. Data are presented as median (95% CI) values.

Hemoglobin polymorphisms	n	Single drug		Combination	
		DHA	PIP	Sum FIC	Drug interaction
Normal hemoglobin	8	4.22 (2.69-6.31)	41.0 (28.4-54.1)	1.051 (1.000-1.125)	No interaction
Hemoglobin E trait	10	3.79 (2.87-5.40)	32.8 (20.5-39.0)	1.106 (1.000-1.464)	No interaction
β-thalassemia trait	2	1.80 (1.54-2.06)	28.2 (24.5-31.9)	1.000 (0.996-1.402)	No interaction
α-thalassemia trait	2	2.79 (2.16-3.42)	57.4 (53.1-61.8)	1.000 (0.889-1.184)	No interaction
Hemoglobin CS trait	1*	2.32 (1.47-3.04)	32.2 (32.1-32.6)	1.000 (0.956-1.642)	No interaction

* Data from triplicate

Table 4. Dihydroartemisinin (DHA) and piperaquine (PIP) IC_{50} (nM) in K1 *P. falciparum*-infected RBCs with normal hemoglobin or hemoglobinopathic RBCs. Data are presented as median (95% CI) values.

Hemoglobin polymorphisms	n	Single drug		Combination	
		DHA	PIP	Sum FIC	Drug interaction
Normal hemoglobin	8	2.74 (2.41-4.09)	63.1 (50.5-78.9)	1.002 (1.000-1.078)	No interaction
Hemoglobin E trait	10	2.71 (1.34-4.66)	56.6 (38.2-94.0)	1.268 (1.000-1.438)	No interaction
β-thalassemia trait	2	3.08 (1.65-4.51)	77.6 (67.8-87.5)	1.252 (1.000-1.428)	No interaction
α-thalassemia trait	2	1.84 (1.61-2.06)	59.3 (50.2-68.5)	0.883 (0.662-1.000)	Additive
Hemoglobin CS trait	1*	0.76 (0.55-1.21)	30.3 (20.2-153.9)	1.000 (0.942-1.296)	No interaction

* Data from triplicate

3.4 Effects of hemoglobinopathic RBCs on *P. falciparum* PI(3)P production following dihydroartemisinin and piperaquine exposure

PI(3)P production in 3D7 *P. falciparum*-infected RBCs with either HbA or hemoglobinopathic RBCs following DHA and PIP exposure was determined and compared with untreated control (Fig. 1). Following DHA exposure, the expression of PI(3)P both decreased and increased compared to untreated samples, as the PI(3)P production ratio was lower or higher than 1. The extent of change was not, however, significant in any type of infected-RBCs (p>0.05). The median (95% CI) changes in PI(3)P expression for parasite-infected HbA, HbE, β-thalassemia trait, α-thalassemia trait, and HbCS trait RBCs were 1.17-(1.00-3.32), 1.03-(1.00-13.19), 3.87-(2.39-5.35), 1.05 (0.36-1.75), and 4.31-fold, respectively. The increase in gene expression seen in 5 of the samples was higher than the cut-off value of 2.5-fold. These 5 samples included 2 HbA (3.32- and 3.54-fold, 20%), 1 HbE trait (13.19-fold, 10%), 1 β-thalassemia trait (5.35-fold, 50%), and 1 HbCS trait (4.31-fold, 100%) RBC types.

In contrast to DHA, PIP did not affect the production of PI(3)P following a 24-hour exposure. The median (95% CI) fold-change in PI(3)P expression in parasite-infected HbA, HbE trait, β-thalassemia trait, α-thalassemia trait, and HbCS RBCs were 1.10-(0.76-1.51), 1.05-(0.73-1.58), 1.25-(0.97-1.54), 1.07-(0.65-1.49), and 0.54-fold, respectively.

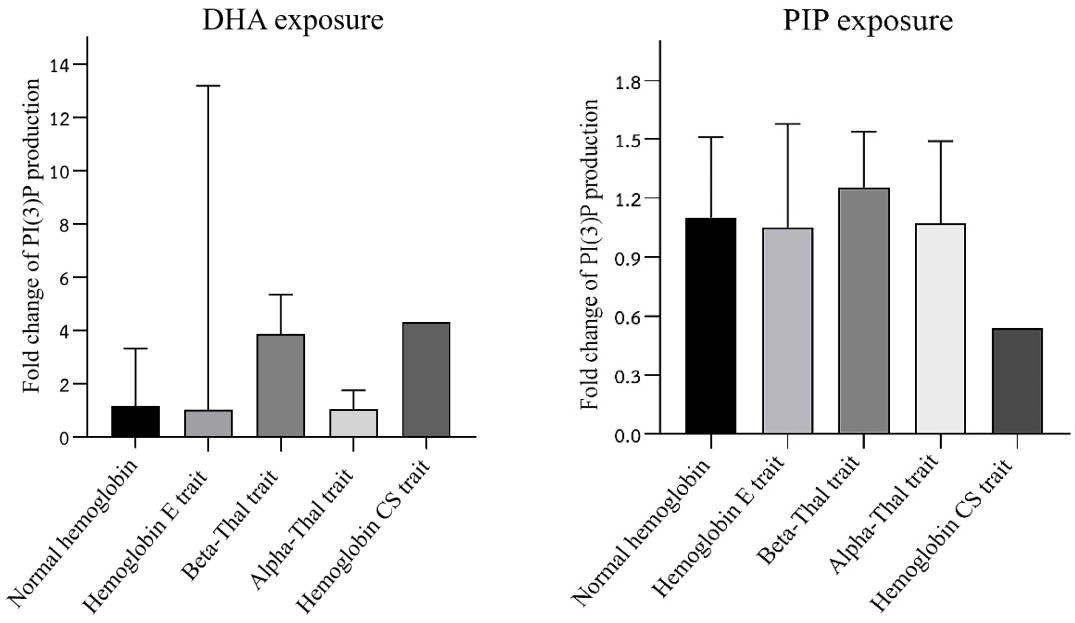

Fig. 1. PI(3)P expression of 3D7 *P. falciparum*-infected RBCs with normal hemoglobin $(n=9)$, hemoglobin E trait $(n=6)$, β-thalassemia trait $(n=2)$, α-thalassemia trait $(n=2)$, and hemoglobin CS trait $(n=1)$ traits following exposure to dihydroartemisinin (DHA) and piperaquine (PIP). Data are presented as median (95% CI).

4. Discussion

RBC proteins, both extracellular and intracellular, are involved in malaria infection and pathogenesis. The infection is initiated by the binding between merozoite-stage parasite proteins and the membrane of a host RBC. Inside RBCs, the parasite's growth is supported by the consumption of globin protein degraded from hemoglobin. Abnormal hemoglobin, resulting from defective production of the α- or β-chains of globin protein, results in poor parasite growth and thus protects the host from the development of complicated malaria [1, 26]. In this study, all hemoglobinopathic RBCs showed slightly different CBC profiles but could be infected by both 3D7 (CQ-sensitive) and K1 (CQ-resistant) *P. falciparum*.

The MCV, MCH, and MCHC values are the parameters that indicate RBC hemoglobin contents and the level of hemoglobinopathy. A mild level of RBC hemoglobinopathy has been reported to be sensitive to malaria infection [27, 28]. The parasite densities following the 144-hour (3 multiplication periods) and 96-hour (2 multiplication periods) incubation periods were generally comparable. It was noted however, that the maximum parasitemia of HbA, HbE trait, β-thalassemia trait, and HbCS trait RBCs was seen at 96 hours of incubation, but that of parasite-infected α- thalassemia trait RBCs was seen at 144 hours of incubation. This confirms that the quality of RBC hemoglobin and membrane proteins may affect parasite growth and multiplication. The maximum parasite growth rate was generally found at 96 hours and gradually declined from there, probably due to insufficient essential nutrients and environmental conditions (the composition of O_2, N_2, and CO_2) [29, 30]. Interestingly, β-thalassemia trait

RBCs were found to be the most resistant to malaria infection.

The ACT, DHA-PIP, is currently recommended by the Ministry of Public Health of Thailand as the first-line treatment for uncomplicated malaria in all malaria endemic areas to overcome multidrug-resistant *P. falciparum* [7, 8]. Variation in the antimalarial response for patients who carried hemoglobinopathic RBCs is of concern. This study showed that 3D7 *P. falciparum*-infected hemoglobinopathic RBCs including -β-thalassemia trait, α-thalassemia trait, and HbCS trait RBCs were more sensitive to DHA than HbA and HbE trait RBCs were. The sensitivity to PIP was highest in parasite-infected β-thalassemic trait RBCs, but among the normal and other hemoglobinopathic RBCs, PIP sensitivity was similar. On the other hand, parasite sensitivity to both DHA and PIP were significantly lower in K1 infected-β-thalassemic trait RBCs compared with K1-infected HbA, HbE trait, α-thalassemia trait, and HbCS trait RBCs. Inappropriate intracellular conditions in RBCs with α-thalassemia trait, HbH [31], HbS, and HbC [32] result in the suppression of parasite growth and poor parasite health, which facilitates elimination by the action of antimalarial drugs. The lower DHA sensitivity seen in parasite-infected HbE RBCs compared to HbA RBCs could be explained by the accumulation of the drug in RBC components, particularly cell membranes [4]. In agreement with a previous report [33], results from the current study suggested no drug interaction between DHA and PIP in both 3D7 and K1 *P. falciparum*-infected RBCs with normal and abnormal hemoglobin; only K1 infected-RBCs with α-thalassemia trait showed an additive effect.

Malaria parasites require essential phospholipids for their survival. Phosphatidylinositol (PI) is synthesized by the parasite through the incorporation of inositol from host serum or *de novo* production [34]. The phosphate group is added to the PI at the carbon atom at position 3 by the phosphatidylinositol-3-kinase (PI3K) enzyme to form PI(3)P. The PI(3)P functions in lipid signaling, cell signaling, and membrane protein trafficking [35]. Enhancing PI(3)P expression, even by as low as 2.5-fold, could significantly impact lipid signaling cascades and induction of drug resistance [16]. Using a 2.5-fold change as the cut-off level for PI(3)P expression, a significant increase in PI(3)P expression was found in 5 RBC samples following DHA exposure. Of them, 2 were parasite-infected RBCs with HbA, and 1 sample each for parasite-infected HbE trait, β-thalassemia trait, and HbCS trait RBCs. No change in PI(3)P expression was observed following PIP exposure.

The increase in PI(3)P production by *Pf*EMP1 transportation (cytoadherence) or *Pf*PI3K activity (*Pf*Kelch13 gene mutation) may, at least in part, be involved in the parasite adaptation process to neutralize artemisinin toxicity, which leads to parasite resistance to DHA. Over-expression of PI(3)P, therefore, promotes development of resistance of *P. falciparum* to artemisinin drugs even without the involvement of gene mutation [36]. Elevation of oxidative stress components, in serum collected from hemoglobinopathic individuals such as those with thalassemia or HbE enhances malaria parasite adaptation of their biological processes for their survival, including induction of mutation of the *Pf*Kelch13 gene to overcome the environmental oxidative stress [37]. For PIP, even the mechanism of parasite resistance to this combination partner is not related to DHA resistance but selective drug pressure from multidrug-resistant antimalarial drugs, including DHA in Southeast Asia, can accelerate parasite resistance development to PIP [38].

5. Conclusion

Results of this study suggest that host hemoglobinopathies slightly affect the susceptibility of *P. falciparum* to DHA and PIP

as well as the production of PI(3)P after DHA exposure. However, treatment response following the DHA- PIP combination therapy may be variable depending on hemoglobin variants. Treatment failure may lead to selective drug pressure and the development of resistance of the parasite to DHA and PIP, and therefore, monitoring the clinical efficacy of DHA- PIP combination therapy should be regularly performed.

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