RESEARCH NOTE

EFFECT OF CHOLINE KINASE INHIBITOR HEXADECYLTRIMETHYLAMMONIUM BROMIDE ON *PLASMODIUM FALCIPARUM* GENE EXPRESSION

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Abstract. Investigations on the fundamental of malaria parasite biology, such as invasion, growth cycle, metabolism and cell signalling have uncovered a number of potential antimalarial drug targets, including choline kinase, a key enzyme involved in the synthesis of phosphatidylcholine, an important component in parasite membrane compartment. The effect on gene expression of *Plasmodium falciparum* K1 strain following 72 hours exposure to 2 μM (IC_{50} concentration) of the choline kinase inhibitor, hexadecyltrimethylammonium bromide (HDTAB) was evaluated by DNA microarray analysis. Genes important in *P. falciparum* intra-erythrocytic life cycle, such as invasion, cytoadherance and growth were among those affected by at least 2-fold changes in their expression levels compared with non HDTAB-treated control.

Keywords: *Plasmodium*, antimalarial, choline kinase

INTRODUCTION

The search for protein kinase inhibitor as new antimalarial agents is a great interest in malaria drug discovery research. *Plasmodium falciparum*, the most deadly human malaria parasite, shares a similar phylogenetic tree of metazoan protein kinase related enzymes (Hanks, 2003). Some of the kinases show quite distinct behavior in terms of their regulation, including the absence of certain kinases, which emphasises the existence of novel pathway that could be exploited for drug target intervention (Doerig *et al*, 2008). The identification of many plasmodial protein kinases shows the importance of protein phosphorylation in *Plasmodium* life cycle, including cell cycle, cell proliferation and differentiation, parasite egress, invasion and host-parasite interaction (Chung *et al*, 2009). Recently, phosphoproteome studies have identified over 5,000 protein phosphorylation sites on *P. falciparum* proteins (Solyakov *et al*, 2011; Treeck *et al*, 2011).

One of the potential drug target candidates is *P. falciparum* choline kinase, an

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essential enzyme in the choline pathway to produce phosphatidylcholine (PC), which is the major phospholipid in eukaryotic membranes (Kent, 1990). Other than that, PC is also important in other functions, such as in the transportation of cholesterol throughout the organism, acting as a substrate for production of second messenger and as a cofactor for the activity of several membrane related enzymes. Choline kinase is also important in the production of sphingomyelin, a membrane phospholipid, and in the regulation of cell growth (Kent, 1990). Malaria parasite synthesizes choline kinase *de novo* (Ancelin and Vial, 1986a) and plays an important role in the growth and multiplication of *P. falciparum* during asexual red blood cell cycle (Choubey *et al*, 2006). Infection with *Plasmodium* increases choline kinase activity in host red blood cells showing that *Plasmodium* contributes to a large proportion of choline kinase activity in infected red blood cells, hence increasing the possibility of choline kinase as a potential antimalarial target (Ancelin and Vial, 1986a). In addition, protein sequence analysis of *P. falciparum* choline kinase (PfCK) and human choline kinase shows some dissimilarity especially in the N-terminal region (Choubey *et al*. 2006). Furthermore, PfCK is lethal in gene knockout experiment emphasizing that this enzyme is essential to the malaria parasite (Dechamps *et al*, 2010).

Quartenary ammonium compounds such as hemicholinium (HC3), a known mammalian choline kinase inhibitor, has been shown to exhibit an inhibitory effect on *P. falciparum* growth *in vitro* (Ancelin *et al*, 1985; Ancelin and Vial, 1986b). Another choline kinase inhibitor, hexadecyltrimethylammonium bromide (HDTAB) has been shown to specifically inhibit PfCK activity (Choubey *et al*, 2007). It has also been evaluated for its antimalarial activity in a mouse model (Choubey *et al*, 2007). However, the effect of HDTAB on malaria parasite gene expression levels has not been investigated.

This study evaluated the effect of HDTAB on the levels of *P. falciparium* gene expression as analysed by DNA microarray.

MATERIALS AND METHODS

In vitro cultivation of *P. falciparum*

Continuous cultivation of malaria parasite, *P. falciparum in vitro* was maintained according to the candle jar method of Trager and Jensen (1976) in complete culture medium containing RPMI 1640 (Gibco, Gaithersberg, MD), 0.2% sodium bicarbonate, 25 mM HEPES, 40 µg/l gentamicin and 10% human serum. Parasites were grown in fresh "O" type human erythrocytes at 7% hematocrit and were synchronized at the ring stages by the procedure of Lambrose and Vanderberg (1979).

In vitro inhibitory assay of HDTAB

Antiplasmodial drug sensitivity assay was based on the measurement of the histidine rich protein II (HRPII) as described by Noedl *et al* (2002). In brief, stock HDTAB (Sigma, St. Louis, MO) dissolved in DMSO was two-fold diluted in water and 10 µl aliquots were added into 96-well plates (concentrations ranging from 1.6 to 100 µM, and final concentration of DMSO < 0.3%) containing 190 µl/well of 0.5% ring-stage parasitized red blood cells (RBCs) at 1.5% hematocrit. Chloroquine (Sigma, St Louis, MO) was used as positive and water as negative control, respectively. For preparation of HRPII ELISA plates, a 100 µl aliquot of IgM capture antibody (MPFM-55A) (ICL, Newberg, OR,) [1 µg/ml in phosphate-

buffered saline (PBS)] was added to each well of a new 96-well plate and incubated at 4ºC overnight. Plates were washed three times with PBS-Tween 20 (PBST) solution, incubated with 200 µl/well of 2% bovine serum albumin in PBS for 2 hours and washed as described above. A 100 µl aliquot of the parasite lysate (thawed from the cell suspension kept at -80ºC) was added to each well of the ELISA plate, followed by incubation for 1 hour at room temperature. Plates were washed as described above and 100 µl aliquot of the detector antibody (MPFG-55P) (ICL, Newberg, OR) conjugated to horseradish peroxidase (0.2 µg/ml) was added to each well, followed by another hour of incubation at room temperature. Following a subsequent washing step, 100 µl aliquot of tetramethylbenzidine chromogen (Zymed Lab, South San Francisco, CA) was added to each well for 10 minutes in the dark, followed by the addition of 50 µl aliquot of 1M sulphuric acid to terminate the reaction. Absorbance at 450 nm was measured using ELISA plate reader (Thermo Multiskan EX, Wilmington, DC). IC_{50} value of each drug was determined using HN-Lin software (malaria.farch.net) and presented as mean ± SEM from 2 different experiments, each conducted in duplicate.

Gene expression analysis using Affymetrix DNA microarray system

P. falciparum was exposed to HDTAB at IC_{50} value (2 µM) for 72 hours and then the RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) followed by purification using Purelink Micro to Midi kit (Invitrogen, Carlsbad, CA) and DNase treatment to the manufacturer's instructions. RNA was spiked with eukaryotic poly-A RNA as positive labelling control and was then converted to cDNA using GeneChip® Expression 3'-Amplification Reagent One-Cycle cDNA synthesis kit (Affymetrix, Santa Clara, CA). In brief, RNA (2 µg) was mixed with T7 oligo primer (50 µM) followed by addition of 1st strand master mix (1st strand Reaction mix, 0.1M DTT and 10 mM dNTPs) and incubated at 42ºC for 2 minutes followed by addition of Superscript II and incubated at 42ºC for 1 minute. Then 2nd strand master mix (1X 2nd strand Master mix, 10 mM dNTPs, *E. coli* DNA ligase, *E. coli* DNA polymerase 1 and RNase H) was added and the mixture incubated at 16ºC for 2 hours, followed by addition of T4 DNA polymerase and incubation at 16ºC for 2 hours. The reaction was terminated by addition of 0.5 M EDTA. cDNA was purified using the GeneChip sample cleanup kit (Affymetrix, Santa Clara, CA) and then converted to cRNA and biotinylated by incubating with the IVT reaction master mix (1X IVT labelling buffer, IVT labelling NTP mix and IVT labelling enzyme mix) (GeneChip® Expression 3'-Ampification Reagent for IVT labelling kit; Affymetrix, Santa Clara, CA) at 37ºC for 16 hours. Biotinylated cRNA was purified using GeneChip® Sample Cleanup Module kit and 20 µg were fragmented by incubating with fragmenting buffer at 94ºC for 35 minutes, then mixed with hybridization cocktail [50 pM control oligonucleotide B2, 1X eukaryotic hybridization controls (bioB, bioC, bioD, cre), 1X hybridization mix and 10% DMSO] prior to hybridization onto GeneChip® *Plasmodium*/*Anopheles* Genome Array (Affymetrix, Santa Clara, CA) and scanned by Affymetric GeneChip Scanner 3000 equipped with GeneChip® Operating Software (GCOS) and GeneChip Fluidics Station 450. The DNA microarray data were then analysed using Partek Genomic Suite software 6.6 (Partek, St Louis, MO). All microarray

data have been deposited in NCBI Gene Expression Omnibus (GEO) database (accession no. GSE54775).

RESULTS

In vitro inhibitory assay of HDTAB

Using the Histidine Rich Protein II assay, IC_{50} value of HDTAB and chloroquine tested in a 72 hour culture of *P. falciparum* K1 strain was 2.0 ± 1.3 μM and 150 ± 27.2 nM, respectively. The IC_{50} value for HDTAB was within the good antimalarial cut off level (IC_{50}≤5 μM) (Fidock *et al*, 2004). In contrast, due to different approach in previous study conducted by Choubey *et al* (2007), the IC_{50} value of HDTAB was between 5 to 10 μM. As a control drug, the chloroquine scored within the acceptable range of IC_{50} value (≥ 100 nM) against the chloroquine resistant *P. falciparum* strains (Sidhu *et al*, 2002).

DNA microarray analysis

The effect of HDTAB on *P. falciparum* gene expression levels were evaluated using DNA microarray analysis. The expression of 49 *P. falciparum* genes (out of 4,300 genes) was up- and down regulated at least 2 folds in response to HDTAB treatment. All microarray data and details of experimental design were submitted to the NCBI Gene Expression Omnibus (GEO) database and are delivered under series accession number GSE54775. Analysis of the functions of these genes using the malaria parasite metabolic pathway database (http://priweb.cc.huji.ac.il/malaria/) and genomic database (http://plasmodb.org/plasmo/), showed that the majority of the down-regulated genes are involved in parasite invasion, motility, post-translational modification and protein translation (Table 1). Other down-regulated genes involved amino acid synthesis, nucleotide synthesis, protein trafficking, lipid metabolism and mitosis and chromosome separation.

HDTAB treatment also increased expression by at least 2 folds of parasite genes involved in motility, lipid metabolism, cell-cell interaction, mitosis and chromosome separation and organellar function (Table 2). In addition, there were 6 genes of unknown functions affected by HDTAB treatment, such as Cof-like hydrolase, had-superfamily, subfamily2b (PF3D7_1226300), *Plasmodium* exported protein (PHISTb) (PF3D7_0702100), plasmepsin X (PF3D7_0808200), merozoite adhesive erythrocyte binding protein (MAEBL) (PF11_0486), steroid dehydrogenase (PFD1035w) and asparagine-rich protein (PFI1520w) (Tables 1 and 2).

DISCUSSION

The broad effect of HDTAB treatment on the *P. falciparum* gene expression profile is not unexpected because the parasites were exposed to IC_{50} value of HDTAB (2 μM) for 72 hours. All of the genes down-regulated by HDTAB are involved in processes during early trophozoite to schizont transition in red blood cells. For example, the components for biochemical processes that are expressed in early trophozoite stage such as transcription and translational machinery (basic transcription factor 3b and 60S ribosomal protein L27, L26, and elongation factor G) (Bozdech *et al*, 2003) were down-regulated in this study (Table 1). The HDTAB treatment also affected the transition of trophozoite to schizont stage as the biochemical markers for DNA replication such as DHFR-TS (Bozdech *et al*, 2003) was also down-regulated (Table 1). Also as some of the affected genes are involved in invasion (coronin, Myosin A, membrane skeletal IMC1, EBA140 and others) (Table 1),

Table 1
P. falciparum genes down-regulated following 72 hours exposure to 2 μM HDTAB.

Gene ID	Gene name	Expression[a] (fold change)
Invasion and motility		
PF3D7_1301600	Erythrocyte binding antigen-140 (EBA-140)	2
PF3D7_0102500	Erythrocyte binding antigen-181(EBA-181)	2
PF3D7_1136900	Subtilisin-like protease 2 (SUB-2)	2
PF3D7_1252400	Reticulocyte-binding protein 3 homologue (Rh3)	2
PF3D7_1251200	Coronin	3
PF3D7_1335300	Reticulocyte binding protein 2 homolog B (Rh2b)	2
PF3D7_0304100	Membrane skeletal protein IMC1-related (ALV2)	3
PF3D7_0424200	Reticulocyte binding protein homolog 4 (Rh4)	2
PF3D7_1342600	Myosin A	2
Post translational modification		
PF3D7_1017900	26 S proteasome subunit p55	2
PF3D7_1138500	Protein phosphatase 2C	3
PF11_0273	DNAJ protein	4
PF3D7_1237000	Ubiquitin activating enzyme (Uba2)	2
PF3D7_1238900	Calcium/calmodulin-dependent protein kinase 2 (PK2)	2
PF3D7_1311800	M1-family alanyl aminopeptidase (M1AAP)	4
PF3D7_1423300	Serine/threonine protein phosphatase (PP7)	2
PF3D7_0214600	Serine/threonine protein kinase	2
PF3D7_0727400	Proteasome subunit alpha type 5	4
PF3D7_0821800	Sec61 beta subunit	4
PF3D7_0810300	Protein phosphatase	3
PF3D7_0823300	Histone acetyltransferase GCN5	2
Gene transcription and translation		
PF3D7_1126200	40 S ribosomal protein S18	2
PF3D7_1351400	60 S ribosomal protein L17	2
PF3D7_1426100	Basic transcription factor 3b	4
PF3D7_1460700	60 S ribosomal protein L27	3
PF3D7_0312800	60 S ribosomal protein L26	3
PF3D7_0315100	Translation initiation factor 4E (eIF4E)	2
PF3D7_0322900	40 S ribosomal protein S3A	2
PF3D7_0416100	Glutamyl-tRNA (Gln) aminotransferase subunit A	2
PF3D7_0602400	Elongation factor G	3
Others		
PF3D7_1129000	Spermidine synthase	2
PF3D7_1226300	Cof-like hydrolase, had-superfamily, subfamily2b	2
PF3D7_1412000	P1/s1 nuclease	2
PF3D7_0417200	Bifunctional dihyfrofolate reductase-thymidylate synthase (DHFR-TS)	3
PF3D7_0702100	*Plasmodium* exported protein (PHISTb)	2
PF3D7_0822600	Pfsec23	2
PF3D7_0808200	Plasmepsin X	3
PF3D7_0904700	Bacterial histone-like protein	2
PF3D7_0924000	Patatin-like phospholipase	3

Genes are identified using PlasmoDB database (www.plasmodb.org) as indicated by gene ID.
[a]Relative to non HDTAB-treated control.

Table 2
P. falciparum genes up-regulated following 72 hours exposure to 2 μM HDTAB.

Gene ID	Gene name	Expression[a] (fold change)
PF10_0275	Protoporphyrinogen oxidase	3
PF11_0486	Merozoite adhesive erythrocyte binding protein (MAEBL)	2
PFD1035w	Steroid dehydrogenase	2
PF3D7_0315200	CSP and TRAP-related protein (CTRP)	3
PFE1430c	Cyclophilin (CYP23)	2
PF10_0397	Rifin	2
PF3D7_0615100	Enoyl-acyl carrier reductase	4
PFI1520w	Asparagine-rich protein	3
PFD1050w	Alpha tubulin 2	3
PFL2405c	PFG 377	3

Genes are identified using PlasmoDB database (www.plasmodb.org) as indicated by gene ID.
[a]Relative to non HDTAB-treated control.

HDTAB would compromise entry of the parasites into the 2nd growth cycle. Thus it is not surprising that gene knockout of PfCK is lethal to *P. falciparum* blood stages (Dechamps *et al*, 2010).

Interestingly, instead of down-regulation, HDTAB treatment had also up-regulated a number of parasite genes. One possible explanation is that the malaria parasite has an ability to change its metabolism in order to adapt to different environment stress conditions, including those imposed by drugs (Ganesan *et al*, 2008; Le Roch *et al*, 2008; Kritsiriwuthinan *et al*, 2011). Other than HDTAB, the antimalarial drugs such as chloroquine, pyronaridine (CQ-related drug) and antifolate (WR99210) also induced changes in expression of host-exported proteins of multigene families such as rifin (Table 2) (Ganesan *et al*, 2008; Kritsiriwuthinan *et al*, 2011). Although the function of rifin is not well understood, the regulation of rifin expression upon chemical stress could be the survival mechanism of *P. falciparum*. It has also been proposed that cell cycle arrest and stimulation of malaria parasite sexual development (gametogenesis) are initiated upon chemical stress (Deitsch *et al*, 2007). Indeed, some of the upregulated gene has been found to be specifically expressed in gametocyte stages. These includes α-tubulin 2 (male gametocyte marker), PFG 377 (female gametocyte marker) and metabolic enzymes such as protoporphyrinogen oxidase and enoyl-acyl carrier reductase (Table 2) (Florens *et al*, 2002; Young *et al*, 2005). Shifting from blood stage to gametocyte stage will change the order of parasite metabolism in order to adapt the oxygen-rich mosquito sexual stages (Young *et al*, 2005). The mitochondria metabolism will become active hence upregulating the mitochondrion-localized enzyme such as protoporphyrinogen oxidase (Table 2) (Florens *et al*, 2002). Gametocyte specific expression for fatty acid biosynthesis has also been observed (Young *et al*, 2005). This support the present study data where the fatty acid biosynthesis enzyme such as enoyl-acyl carrier reductase was upregulated

in HDTAB treated *P. falciparum* (Table 2).

Similar to other antimetabolites (Ganesan *et al*, 2008; Le Roch *et al*, 2008), HDTAB does not trigger any changes to RNA level of choline kinase and other phosphatidylcholine synthesis related enzymes. However, this phenomenon can only be confirmed by further proteomic analysis.

In summary, our study has demonstrated that PfCK inhibitor HDTAB is a potential candidate for antimalarial drug development. It should be employed in synergistic combination with known or other potential antimalarial drug candidates in order to improve efficacy and effectiveness in parasite clearance and in delaying emergence of drug resistant parasites.

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