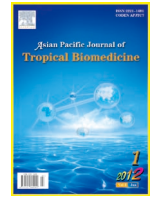




Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.elsevier.com/locate/apjtb



Document heading doi:10.1016/S2221-1691(11)60192-5 © 2012 by the Asian Pacific Journal of Tropical Biomedicine. All rights reserved.

Comparison of protein patterns between *Plasmodium falciparum* mutant clone T9/94–M1–1(b3) induced by pyrimethamine and the original parent clone T9/94

Kanchana Rungsihirunrat¹, Wanna Chaijaroenkul², Napaporn Siripoon¹, Aree Seugorn¹, Sodsri Thaithong¹, Kesara Na–Bangchang^{2*}

¹College of Public Health Sciences, Chulalongkorn University, Bangkok 10330, Thailand

²Graduate Program in Biomedical Sciences, Thammasat University, Patumthanee 12121, Thailand

ARTICLE INFO

Article history:

Received 5 May 2011

Received in revised form 27 May 2011

Accepted 18 June 2011

Available online 28 January 2012

Keywords:

Plasmodium falciparum

Proteomics

Pyrimethamine

Drug resistance

Protein

Molecular target

ABSTRACT

Objective: To compare the protein patterns from the extracts of the mutant clone T9/94–M1–1(b3) induced by pyrimethamine, and the original parent clone T9/94 following separation of parasite extracts by two-dimensional electrophoresis (2-DE). **Methods:** Proteins were solubilized and separated according to their charges and sizes. The separated protein spots were then detected by silver staining and analyzed for protein density by the powerful image analysis software. **Results:** Differentially expressed protein patterns (up- or down-regulation) were separated from the extracts from the two clones. A total of 223 and 134 protein spots were detected from the extracts of T9/94 and T9/94–M1–1(b3) clones, respectively. Marked reduction in density of protein expression was observed with the extract from the mutant (resistant) clone compared with the parent (sensitive) clone. A total of 25 protein spots showed at least two-fold difference in density, some of which exhibited as high as ten-fold difference. **Conclusions:** These proteins may be the molecular targets of resistance of *Plasmodium falciparum* to pyrimethamine. Further study to identify the chemical structures of these proteins by mass spectrometry is required.

1. Introduction

Malaria is still a major public health problem affecting the populations in tropical and subtropical countries[1]. The major cause contributing to failure to eliminate malaria is the emergence and spread of resistance of malaria parasites to most of the available antimalarial drugs. Research and development of new antimalarial agents are therefore urgently needed to overcome the situation. Among the four species of human malaria, *Plasmodium falciparum* (*P. falciparum*) is the most virulent and is responsible for the vast majority of deaths worldwide. The complete genome

sequence of the *P. falciparum* clone 3D7 provides valuable information for proteomics investigation of the parasite in order to identify new potential drug and vaccine targets[2].

Folate metabolism of malaria parasite has been well established as one of the most valuable targets for antimalarial drugs. Antifolate agents such as pyrimethamine, proguanil and chlorproguanil act by inhibiting dihydrofolate reductase (DHFR) enzyme in folate biosynthesis pathway[3]. Resistance to antifolates occurs through stepwise mutations of this enzyme, which eventually causes the change in parasite's sensitivity to the drugs[4,5]. To understand the underlying mechanism of antifolate resistance, Thaithong *et al*[6] obtained the mutant clone T9/94–M1–1(b3) with increased resistance to pyrimethamine through selective drug pressure. The mutant clone T9/94–M1–1(b3) exhibited a 100 fold increase in minimal inhibitory concentration (MIC) of pyrimethamine (5×10^{-6} M) as compared with the original parent clone (5×10^{-8} M). However, no change in the coding sequence of neither DHFR nor gene amplification was observed in both clones. Since the decrease in sensitivity of the mutant clone T9/94–M1–1(b3) could not be explained on the basis of the mutation in the DHFR gene, it is imperative

*Corresponding author: Dr. Kesara Na–Bangchang, Professor, Ph.D. (Pharmacology) Director, Graduate Program in Biomedical Sciences Coordinator, WHO–TDR Clinical Coordination and Training Center Faculty of Allied Health Sciences, Thammasat University (Rangsit Campus), Klongluang, Pathumthani, 12121, Thailand.

Tel: 662 9869213 ext. 7271

Fax: 662 9869207

E-mail: kesaratmu@yahoo.com

Foundation Project: Supported by the Thailand Research Fund (TRF), Rachadaphisek Sompok Research Fund, Chulalongkorn University, the National Research University (NRU) Project of Thailand, CHE–RES Project, Office of Higher Education Commission and Ministry of Education of Thailand.

to ascertain whether the antifolate drugs induce differential changes in the expression levels of other proteins apart from DHFR enzyme. The aim of the present study was to explore other protein targets other than the target enzyme DHFR, which may be involved in resistance of *P. falciparum* to antifolates. Comparative protein patterns of both the sensitive and resistant clones were analyzed after separation by 2-dimensional electrophoresis (2-DE).

2. Materials and methods

2.1. Parasite culture

The *P. falciparum* clone T9/94 used in this study was cultured *in vitro* in group O human red blood cells according to the previously described method [6]. Briefly, parasites were grown in RPMI1640 medium (supplemented with 37.5 mM HEPES, 7 mM D-glucose, 6 mM NaOH, 25 μ g/mL gentamicin sulphate, 2 mM L-glutamine and 10% human serum) under an atmosphere of 96% nitrogen, 3% carbon dioxide, and 1% oxygen. Synchronization of the parasite to early ring stage was performed using 5% sorbitol. The mutant clone T9/94M1-1(b3) with up to 1 000-fold increase in resistance to pyrimethamine was selected by exposing the parent T9/94 clone with stepwise increased concentrations of pyrimethamine in culture medium. The synchronized culture of both the parent and mutant clones were further maintained until approximately 5% parasitemia of schizont were obtained.

2.2. Extraction of parasite protein

Synchronized parasite culture was harvested and cell pellet was resuspended in 0.15% saponin in PBS and incubated on ice for 1 h in order to lyse red cells. The lysate was collected through centrifugation at 13 000 \times g for 5 min (4 $^{\circ}$ C) and washed three times with 1 mL of PBS until the supernatant was clear. Red blood cell pellet was washed in 1 mL of 10 mM Tris-HCl (pH 7.4) containing 1 \times protease inhibitor Cocktail (Roche Co. Ltd.) until red cell ghost was colorless. Pellet was then re-suspended in 500 μ L of lysis buffer (8 M urea, 2 M thiourea, 1% CHAPS, 65 mM DTT, 0.5% ampholyte pH 3–10)

and sample was vortexed and sonicated on ice four times, eight seconds each (21% amplitude, 8 sec, interspersed with 9 sec), followed by centrifugation at 13 000 \times g for 1 h (4 $^{\circ}$ C), and the supernatant was subjected to 2-DE. Quantification of protein concentrations of the extracts was performed by using Bradford reagent (BioRad Co. Ltd.).

2.3. Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis (2-DE) was carried out using a 2D electrophoresis system (BioRad Co. Ltd., USD) according to the manufacturer's recommendation with modifications. The extract of parasite protein (100 μ g) was mixed with rehydration buffer (8 M urea, 1% CHAPS, 15 mM dithiothreitol, 0.001% bromophenol blue). Protein mixture (125 μ L) was applied onto 7 cm IPG strips in an isoelectric focusing (IEF) system (BioRad Co. Ltd., USA), and was subjected to pH gradient (3–10) with an electrical charge using ampholytes as carrier. IEF was performed initially at 250 v for 15 min, followed by 4 000 v for 1 h, and terminated with 4 000–20 000 v-h. The focused strips were equilibrated in 5 mL equilibration solution I (37.5 mM Tris-HCl, pH 6.8, 6 M urea, 20% glycerol, 2% SDS) containing the reducing agent DTT (130 mM) for 10 min, followed by 5 mL equilibration solution II containing iodoacetamide (135 mM) for additional 10 min. The strips were then briefly washed with 1 \times gel running buffer and loaded onto 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) for second dimension separation. The gels were run on 1 \times electrode buffer pH 8.3. After electrophoresis, gels were fixed and stained with silver stain (BioRad Co. Ltd., USA) according to the manufacturer's recommendation. The 2-DE gel images were scanned and analyzed by PDQuestTM software (BioRad Co. Ltd., USA). At least four independent gels were analyzed for each sample. The density of protein spots separated from the extracts of pyrimethamine-sensitive and pyrimethamine-resistant clones were compared as the ratio between the spot density of T9/94 and T9/94M1-1(b3).

3. Results

Proteins extracted from pyrimethamine-sensitive (T9/94)

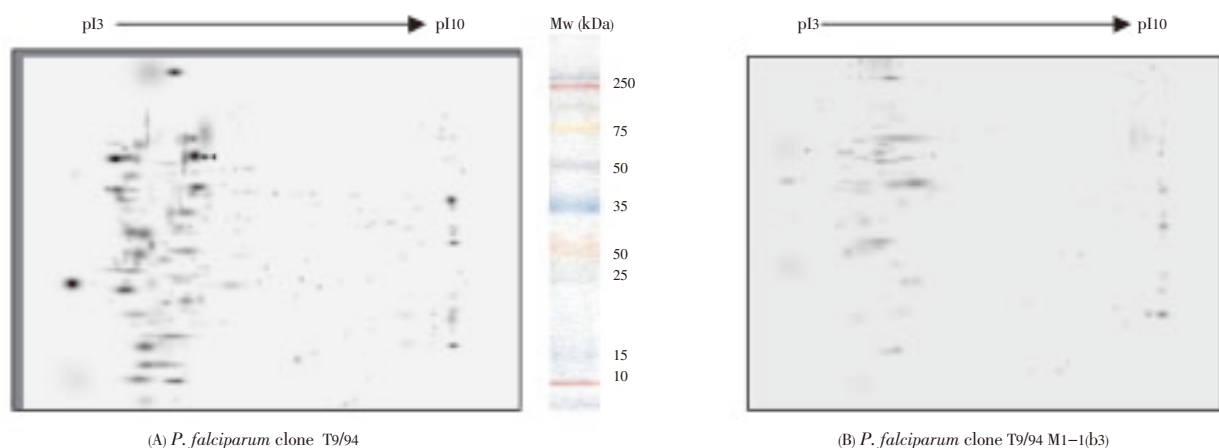


Figure 1. Differential expression of protein spots from the extracts of (A) T9/94, and (B) T9/94 M1-1(b3), separated by 2-DE according to their charges (first dimension by isoelectric focusing IPG strips pH range 3–10), followed by molecular weights (second dimension using 12% SDS-PAGE).

Table 1

Protein spots separated from the extracts of T9/94 (pyrimethamine–sensitive) and T9/94 M1–1(b3) mutant (pyrimethamine–resistant) clones with at least two–fold difference in protein density.

Spot ID	Protein spot density			Spot ID	Protein spot density		
	T9/94	T9/94 M1–1(b3)	Ratio of spot density [T9/94 : T9/94 M1–1(b3)]		T9/94	T9/94 M1–1 b3	Ratio of spot density [T9/94 : T9/94 M1–1(b3)]
SSP0214	9 078.00	2 598.20	3.49	SSP6615	8 567.38	411.97	20.79
SSP2003	15 301.15	1 588.42	9.63	SSP6616	3 501.46	344.86	10.15
SSP2119	15 411.90	205.78	74.89	SSP7111	4 100.69	772.46	5.30
SSP2208	24 868.95	770.26	32.28	SSP7112	1 601.11	135.01	11.85
SSP2313	18 998.45	2 144.37	8.85	SSP7307	1 422.20	216.88	6.55
SSP2614	13 781.72	302.75	45.52	SSP7308	10 054.00	373.10	26.94
SSP2817	4 245.18	674.55	6.29	SSP8507	1 079.04	143.98	7.49
SSP3111	21 031.61	662.08	31.76	SSP9205	5 932.10	2 260.99	2.62
SSP3214	7 075.11	2 539.02	2.78	SSP9206	11 978.64	2 081.70	5.75
SSP3306	9 143.13	604.13	15.13	SSP9405	15 157.39	2 442.15	6.20
SSP4707	16 223.82	3 894.43	4.16	SSP9406	19 770.03	6 221.96	3.17
SSP5713	32 681.61	3 332.01	9.80	SSP9507	19 310.89	4 090.94	4.72
SSP6106	4 944.89	475.05	10.40				

and pyrimethamine–resistant [T9/94M1–1(b3)] *P. falciparum* clones were analyzed by 2–DE. Protein spots were separated from the extracts of both clones using 7 cm immobilized IPG strip at the pH range of 3–10, and were visualized by silver staining gels. The differentially expressed proteins separated by 2–DE gel for each sample were shown in Figure 1. The 2–DE gel images were scanned and analyzed using PDQuest™ software (BioRad Co. Ltd.). The extracts of both clones showed similar basic protein patterns (64% similarity), but with a number of some different spots. A total of 223 and 134 protein spots were detected from the extracts of T9/94 and T9/94–M1–1(b3) clones, respectively. Marked reduction in density of protein expression was observed with the extract from the mutant (resistant) clone compared with the parent (sensitive) clone. A total of 25 protein spots showed at least two–fold difference in density, some of which exhibited as high as ten–fold difference (Table 1).

4. Discussion

Proteomics offers a new approach for exploring potential targets for drug and vaccine development. Traditional methods for characterization and identification of large numbers of proteins from a complex protein mixture have relied mainly on 2–DE combined with mass spectrometry. Numerous proteomics studies of bacterial pathogen as well as other organisms signify the value of these approaches[7,8]. Nevertheless, the application of 2–DE in the investigation of malaria parasite proteomes is still limited. The extraction and solubilization of all components are the critical steps that are essential for the success of the technique. The inefficient extraction procedure, including the large size of some proteins, the contamination of haemoglobin–derived products and thiourea result in low reproducibility of the separated proteins.

Few proteomic research have been undertaken to elucidate the mechanisms of action or resistance of antimalarial drugs in *P. falciparum*[9–11]. In the present study, the proteomic approach was applied to analyze the pattern of protein expression in the schizont stage of *P. falciparum* following being subjected to selective pressure by the antifolate drug

pyrimethamine. Protein expression of entire *P. falciparum* proteome of the pyrimethamine–sensitive and induced resistant clones were compared in order to elucidate potential target(s) of pyrimethamine resistance apart from DHFR enzyme. Although analysis of malaria proteins by 2–DE gel has widely been established, major problem encountered is the insolubility of membrane proteins which leads to reduced efficiency in discriminating the separated protein spots on 2–DE. In addition, contamination by human host proteins with a wide range of molecular weights and isoelectric points (pI) remains problematic for improvement of proteomic profiles. Modification of the extraction protocol based on types of samples, or utilization of a variety of chaotropic mixtures with detergents, has been applied to solve these problems[12–25]. Results from a previous study indicated that approximately 52% of the proteins identified from *P. falciparum* blood stage samples were host red cell proteins[26]. The contamination by haemoglobin–derived products during extraction steps result in smearing and high background after 2–DE separation[27].

The current results of the 2–DE profiles revealed differently expressed proteins separated from the extracts of *P. falciparum* original clone T9/94 and mutant clone T9/94–M1–1(b3). This indicates that the up– or down–regulated proteins may be involved in sensitivity of *P. falciparum* to pyrimethamine. A total of 223 and 134 protein spots, respectively, were detected in T9/94 and T9/94–M1–1(b3) clones. Abundant malaria proteins were mainly distributed in the 2–DE in the pH gradient ranging from 3 to 7, and molecular weights ranging from low to high (10–100 kDa). The low abundant proteins were located at the pH of greater than 7. The protein densities of a total of 25 spots from both clones were found to exhibit at least two–fold difference. Of these protein spots, a 10–fold reduction in protein density was observed in the mutant (resistant) clone compared with the parent (sensitive) clone. The ability to measure the levels of protein expression following drug challenge is one of the main goals in malaria proteomics research. Identification and quantification of proteins from the parasite extracts could be performed in these differentially expressed proteins. Prediction of the chemical structures of these protein spots according to molecular weights and isoelectric

points can be obtained by comparison with those reported in SWISS–PROT database. Nevertheless, in order to clearly elucidate the mechanism of resistance of *P. falciparum* to pyrimethamine, further identification of these proteins by either peptide mass fingerprinting using MALDI–TOF or direct peptide sequencing by tandem mass spectrometry is required. In addition, the optimized 2–DE method with high reproducibility and sensitivity is essential to allow for the highly expedient analysis of differentially expressed malaria proteins at low amounts.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

The study was supported by the Thailand Research Fund (TRF), Rachadaphisek Sompok Research Fund, Chulalongkorn University, the National Research University (NRU) Project of Thailand, CHE–RES Project, Office of Higher Education Commission and Ministry of Education of Thailand.

References

- [1] Guerra CA, Snow RW, Hay SI. Mapping the global extent of malaria in 2005. *Trends Parasitol* 2006; **22**: 353–358.
- [2] Florens L, Washburn MP, Raine JD, Anthony RM, Grainger M, Haynes JD, et al. A proteomic view of the *Plasmodium falciparum* life cycle. *Nature* 2002; **419**: 520–526.
- [3] Bzik DJ, Li WB, Horii T, Inselburg J. Molecular cloning and sequence analysis of the *Plasmodium falciparum* dihydrofolate reductase–thymidylate synthase gene. *Proc Natl Acad Sci USA* 1987; **84**: 8360–8364.
- [4] Gregson AL, Plowe CV. Mechanism of resistance of malaria parasite to antifolates. *Pharmacol Rev* 2005; **57**: 117–145.
- [5] Plowe CV, Djimde A, Bouare M, Doumbo O, Wellems TE. Pyrimethamine and proguanil resistance–conferring mutations in *Plasmodium falciparum* dihydrofolate reductase: polymerase chain reaction methods for surveillance in Africa. *Am J Trop Med Hyg* 1995; **52**: 565–568.
- [6] Thaithong S, Chan SW, Songsomboob S. Pyrimethamine resistance mutations in *Plasmodium falciparum*. *Mol Biochem Parasitol* 1992; **52**: 149–158.
- [7] Cash P. Proteomics of bacterial pathogens. *Adv Biochem Eng Biotechnol* 2003; **83**: 93–115.
- [8] Washburn MP, Wolters D, Yates JR. Large–scale analysis of the yeast proteome by multidimensional protein identification technology. *Nat Biotechnol* 2001; **19**: 242–247.
- [9] Cooper RA, Carucci DJ. Proteomic approaches to studying drug targets and resistance in *Plasmodium*. *Curr Drug Targets Infect Disord* 2004; **4**: 41–51.
- [10] Makanga M, Bray PG, Horrocks P, Ward SA. Towards a proteomic definition of CoArtem action in *Plasmodium falciparum* malaria. *Proteomics* 2005; **5**: 1849–1858.
- [11] Prieto JH, Koncarevic S, Park SK, Yates J, Becker K. Large–scale differential proteome analysis in *Plasmodium falciparum* under drug treatment. *PLoS One* 2008; **3**: e4098.
- [12] Pantumthong P, Vattanaviboon P. Improvement of proteomic profile of *Plasmodium falciparum* by two step protein extraction in two dimensional gel electrophoresis. *Thammasat Int J Sci Technol* 2006; **11**: 61–68.
- [13] Gbotosho GO, Okuboyejo TM, Happi CT, Sowunmi A. *Plasmodium falciparum* hyperparasitaemia in Nigerian children: epidemiology, clinical characteristics, and therapeutic responses to oral artemisinin–based combination treatments. *Asian Pac J Trop Dis* 2011; **1**(2): 85–93.
- [14] Jombo GTA, Araoye MA, Damen JG. Malaria self medications and choices of drugs for its treatment among residents of a malaria endemic community in West Africa. *Asian Pac J Trop Dis* 2011; **1**(1): 10–16.
- [15] George P, Alexander LM, Shetty A. Study comparing the clinical profile of complicated cases of *Plasmodium falciparum* malaria among adults and children. *Asian Pac J Trop Dis* 2011; **1**(1): 35–37.
- [16] Jombo GTA, Alao OO, Araoye MO, Damen JG. Impact of a decade–long anti–malaria crusade in a West African community. *Asian Pac J Trop Dis* 2011; **1**(2): 100–105.
- [17] Alaya–Bouafif NB, Chahed MK, Bez HE, Bellali H, Ayari L, Achour N. Completeness of malaria notification in Tunisia assessed by capture recapture method. *Asian Pac J Trop Dis* 2011; **1**(3): 187–191.
- [18] Osonuga OA, Osonuga AA, Osonuga IO, Osonuga A, Kwarteng DL. Prevalence of hypoglycemia among severe malaria children in a rural African population. *Asian Pac J Trop Dis* 2011; **1**(3): 192–194.
- [19] Gbotosho GO, Okuboyejo TM, Happi CT, Sowunmi A. Recrudescence *Plasmodium falciparum* infections in children in an endemic area following artemisinin–based combination treatments: Implications for disease control. *Asian Pac J Trop Dis* 2011; **1**(3): 195–202.
- [20] Fan Z, Zhang L, Yan G, Wu Q, Gan X, Zhong S, et al. Bioinformatics analysis for structure and function of CPR of *Plasmodium falciparum*. *Asian Pac J Trop Med* 2011; **4**(2): 85–87.
- [21] Ibrahim EA, Kheir MM, Elhardello OA, Almahi WA, Ali NI, Elbashir MI, et al. Cortisol and uncomplicated *Plasmodium falciparum* malaria in an area of unstable malaria transmission in eastern Sudan. *Asian Pac J Trop Med* 2011; **4**(2): 146–147.
- [22] Khan HM, Shujatullah F, Ashfaq M, Raza A. Changing trends in prevalence of different *Plasmodium* species with dominance of *Plasmodium falciparum* malaria infection in Aligarh (India). *Asian Pac J Trop Med* 2011; **4**(1): 64–66.
- [23] Tangpukdee N, Wai KM, Muangnoicharoen S, Kano S, Phophak N, Tiemprasert J, et al. Indicators of fatal outcome in severe *Plasmodium falciparum* malaria: a study in a tertiary–care hospital in Thailand. *Asian Pac J Trop Med* 2010; **3**(11): 855–859.
- [24] Raewadee W, Wanna C, Poonuch M, Prapichaya P, Na–Bangchang K. Polymorphisms of the oxidant enzymes glutathione S–transferase and glutathione reductase and their association with resistance of *Plasmodium falciparum* isolates to antimalarial drugs. *Asian Pac J Trop Med* 2010; **3**(9): 673–677.
- [25] Nmorsi OPG, Isaac C, Ohaneme BA, Obiazi HAK. Pro–inflammatory cytokines profiles in Nigerian pregnant women infected with *Plasmodium falciparum* malaria. *Asian Pac J Trop Med* 2010; **3**(9): 731–733.
- [26] Johnson JR, Florens L, Carucci DJ, Yates JR. Proteomics in malaria. *J Proteome Res* 2004; **3**: 296–306.
- [27] Sims PFG, Hyde JE. Proteomics of the human malaria parasite *Plasmodium falciparum*. *Expert Rev Proteomics* 2006; **3**: 87–95.