VIRTUAL SCREENING AND ENZYMATIC INHIBITION ANALYSIS OF BACTERIALLY EXPRESSED LACTATE DEHYDROGENASE FROM *PLASMODIUM KNOWLESI* FOR ANTIMALARIAL DRUGS DEVELOPMENT

BY

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A thesis submitted in fulfilment of the requirement for the degree of Master of Science (Biotechnology Engineering).

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SEPTEMBER 2021

ABSTRACT

Malaria is a mosquito-borne tropical disease caused by parasite of the Plasmodium genus. Globally, about 229 million cases of malaria with approximately 409 000 mortalities occurred in 2019. In Malaysia, *Plasmodium knowlesi* has now become the most common cause of malaria in the country. Disease management remains challenging due to malaria parasites resistance towards the current antimalarial agents, which is one of the factors that prevent the elimination of malaria. Hence, new drugs to treat malaria specifically caused by *P. knowlesi* should be a priority in malaria research. Selection of antimalarial candidates via virtual screening, which specifically targets enzymes such as lactate dehydrogenase (LDH) in glycolytic pathway of Plasmodium is a good strategy because it is the sole energy producer during erythrocytic cycle of the parasites. The aim of this study is to screen for novel antimalarial candidates *via virtual* screening and to evaluate the effects of the selected virtually screened compounds on the activity of LDH by inhibition studies. The potential compounds were screened *via* computational approach comprising Ligand-Based Drug Design using Ultra-Fast Shape Recognition with Atom Types (UFSRAT) and Ultra-Fast Shape Recognition with CREDO Atom Types (USRCAT), followed by structure-based drug design using Autodock4 programme. UFSRAT have resulted with similarity scores ranged from 0.832-0.914 for compounds that most resemble oxamate, a known LDH inhibitor. Meanwhile, USRCAT similarity scores for compounds that most resemble pyruvate (substrate) and lactate (product), ranged from 0.859 to 0.882 and 0.822 to 0.849, respectively. Structure-based drug design for analogues of oxamate, pyruvate and lactate have resulted with minimum binding energies ranged from -3.59 kcal/mol to -0.07 kcal/mol, -5.25 kcal/mol to -1.99 kcal/mol and -3.74 kcal/mol to -2.81 kcal/mol respectively. Meanwhile, cloning of *P.knowlesi* lactate dehydrogenase (*Pk*-LDH) gene into expression vector (pET21a) was performed, prior to protein expression in bacterial system. SDS-PAGE analyses revealed that a fusion protein of ~34 kDa was present in soluble fraction, which confirmed size of the bacterially-expressed *Pk*-LDH and then was subsequently purified to homogeneity using Immobilized Metal Ion Affinity Chromatography (IMAC) and Size Exclusion Chromatography (SEC). Protein characterization was performed to verify the identity of purified proteins using Matrix-Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass spectrometry, results confirmed that bacterially-expressed *Pk*-LDH obtained in this study is similar to *P. knowlesi* LDH with 282 protein sequence coverage. The structural properties of *Pk*-LDH protein were investigated by using far UV Circular Dichroism (CD) Spectroscopy, where proper folding and correct disulfide linkages in the pure *Pk*-LDH enzyme were verified. The specific activity of the purified *Pk*-LDH enzyme was found to be 475.6 U/mg, confirming the presence of pure and active protein. Finally, selected compounds obtained from *in silico* screening were tested on purified *Pk*-LDH *via* enzymatic inhibition assay and the compound namely oxalic acid has shown 54.12% of inhibition with an IC_{50} value of 0.6398 mM,the most potent compared to other compounds. This study has therefore resulted in the development of a reliable method for producing soluble *Pk*-LDH that is biologically-active and leads to further exploration of the potential compounds that can be developed as promising antimalarial drugs, specifically to treat malaria infections caused by *P. knowlesi.*

خلاصة البحث

الملاريا مرض استوائي ينقله البعوض ويسببه طفيلي من جنس المتصورة. على الصعيد العالمي، حدثت حوالي 229 مليون حالة إصابة بالملاريا مع ما يقرب من 409،000 حالة وفاة في عام 2019م. وفي ماليزيا ، أصبحت المتصورة النولسية الآن السبب الأكثر شيوعًا للملاريا في البلاد. لا تزال إدارة المرض صعبة بسبب مقاومة طفيليات الملاريا للعوامل المضادة للملاريا الحالية، والتي تعد أحد العوامل التي تمنع القضاء على الملاريا. ومن ثم، يجب أن تكون الأدوية الجديدة لعلاج الملاريا التي تسببها المتصورة النولسية على وجه التحديد أولوية في أبحاث الملاريا. يعد اختيار المواد المرشحة المضادة للملاريا عبر الفحص الافتراضي، والذي يستهدف على وجه التحديد الإنزيمات مثل اللاكتات ديهيدروجينيز (LDH) في مسار التحلل الجلدي للمتصورة، استراتيجية جيدة لأنه المنتج الوحيد للطاقة خلال دورة كريات الدم الحمراء للطفيليات. الهدف من هذه الدراسة هو الكشف عن مرشحات جديدة لمضادات الملاريا من خلال الفحص الافتراضي وتقييم تأثيرات المركبات المختارة عمليًا على نشاط LDH من خلال دراسات التثبيط. تم فحص المركبات المحتملة من خلال نهج حسابي يشتمل على تصميم دواء يعتمد على Ligand باستخدام التعرف على الشكل فائق السرعة مع أنواع الذرة (UFSRAT) والتعرف الفائق السرعة على الشكل باستخدام CREDO Atom Types (USRCAT) ، متبوعًا بتصميم عقار قائم على الهيكل باستخدام برنامج Autodock4. نتج عن UFSRAT درجات تشابه تراوحت بين 0.832-0.914 للمركبات التي تشبه الأكسامات إلى حد كبير، وهو مثبط LDH المعروف. وفي الوقت نفسه، تراوحت درجات تشابه USRCAT للمركبات التي تشبه البيروفات (الركيزة) واللاكتات (المنتج) من 0.859 إلى 0.882 و 0.822 إلى 0.849 على التوالي. نتج عن تصميم الأدوية المعتمد على الهيكل لنظائر الأكسامات والبيروفات واللاكتات طاقات الربط الدنيا تتراوح من -3.59 كيلو كالوري / مول إلى -0.07 كيلو كالوري / مول ، و -5.25 كيلو كالوري / مول إلى -1.99 كيلو كالوري / مول و -3.74 كيلو كالوري / مول إلى -2.81 كيلو كالوري / مول على التوالي. وفي الوقت نفسه، تم إجراء استنساخ جين *P.knowlesi* lactate dehydrogenase (*Pk*-LDH) إلى ناقل التعبير (pET21a)، قبل التعبير عن البروتين في النظام البكتيري. كشفت تحليلات SDS-PAGE أن بروتينًا اندماجيًا يبلغ تقريبًا 34 كيلو دالتون كان موجودًا في جزء قابل للذوبان، مما أكد حجم *Pk*-LDH المعبر عنه بالبكتيريا ثم تمت تنقيته لاحقًا للتجانس باستخدام كروماتوجرافيا تقارب أيون معدني غير متحرك (IMAC) وكروماتوجرافيا استبعاد الحجم. (SEC). تم إجراء توصيف البروتين للتحقق من هوية البروتينات المنقاة باستخدام مطياف كتلة التأين لوقت الطيران بمساعدة المصفوفة (MALDI-TOF) ، وأكدت النتائج أن *Pk*-LDH المعبر عنه بالبكتيريا والذي تم الحصول عليه في هذه الدراسة يشبه LDH *P. knowlesi* مع 282 تغطية تسلسل بروتين. تم فحص الخواص الهيكلية لبروتين *Pk*-LDH باستخدام التحليل الطيفي ثنائي اللون الدائري (CD) للأشعة فوق البنفسجية البعيدة، حيث تم التحقق من الروابط الصحيحة للطي والثاني كبريتيد في إنزيم *Pk*-LDH النقي. وجد أن النشاط النوعي لإنزيم *Pk*-LDH المنقى هو 475.6 U / mg، مما يؤكد وجود البروتين النقي والنشط. أخيرًا، تم اختبار المركبات المختارة التي تم الحصول عليها من الفرز السيليكو على-*Pk* LDHالمنقى عن طريق مقايسة التثبيط الإنزيمي وأظهر المركب وهو حمض الأكساليك 54.12٪ من التثبيط بقيمة IC50 تبلغ 0.6398 ملي مولار، وهو الأكثر فعالية مقارنة بالمركبات الأخرى. وبالتالي، أدت هذه الدراسة إلى تطوير طريقة موثوقة لإنتاج *Pk*-LDH القابل للذوبان والذي يكون نشطًا بيولوجيًا ويؤدي إلى مزيد من الاستكشاف للمركبات المحتملة التي يمكن تطويرها كأدوية مضادة للملاريا الواعظة، خاصة لعلاج عدوى الملاريا التي تسببها المتصورة النوليسية.

APPROVAL PAGE

I certify that I have supervised and read this study and in my opinion. It conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Science (Biotechnology Engineering)

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DECLARATION

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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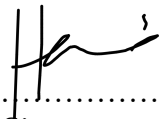

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“Keep your grit and persistent.”

ACKNOWLEDGEMENT

All glory is due to Allah, the Almighty, whose Grace and Mercies have been with me throughout the duration of my study. His Mercies and Blessings on me ease the enormous task of finishing this thesis.

I am most thankful to my supervisor, Asst. Prof. Dr. Fazia Adyani binti Ahmad Fuad, whose continuing compassion, diligence and friendship have facilitated the successful accomplishment of my work. I put on highest appreciation on her comprehensive remarks and inspiring queries which have significantly enhanced this thesis. Her brilliant grasp of the aim and content of this work led to her comments, ideas and queries which helped me a great deal. Despite her commitments, she took time to listen and attend to me whenever requested. The good support she gave me is a boost that helped me in structure and inscription the draft of this research work. I am also thankful to my co-supervisor, Prof. Dr. Azura binti Amid, whose provision and assistance contributed to the outcome of this work.

Lastly, my appreciation goes to my beloved parents, family, friends and colleagues in my workplace in Parasitology Unit, Institute for Medical Research especially to my Head of Unit, Dr. Shamilah Hisam, my colleagues Ms Adela Ida Jiram and Dr. Noor Azian Md Yusuf; for their prayers, understanding and endurance while I am working on this project. Thank you all.

Alhamdulillah.

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LIST OF SYMBOLS

K_m	Michaelis constant
s	Substrate concentration
U	Units
v	Reaction rate
V_{max}	Maximum rate of reaction

LIST OF ABBREVIATIONS

ACT	Artemisinin Combination Therapy
AL	Artemether-lumefantrine
ATP	Adenosine triphosphate
APS	Ammonium Persulfate
CADD	Computer aided drug design
CAS	Chemical Abstracts Service
CD	Circular Dichroisms
CGI	Common gateway interface
cDNA	Complementary DNA
CV	Column volumes
DHFR	Dihydrofolate reductase
dH_2O	Distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate
EDULISS	EDinburgh University Ligand Selection System
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G3P	Glyceraldehyde-3-phosphate
G6PD	Glucose-6-phosphate dehydrogenase
GRAVY	Grand average hydropathicity
hGAPDH	Human Glyceraldehyde 3-phosphate dehydrogenase
HTS	High Throughput screening
IC_{50}	50% inhibitory concentration
IMAC	Immobilised Metal Affinity Chromatography
IPTG	Isopropyl β-D-1-thiogalactopyranoside
K13-propeller	Kelch propeller
LB	Luria Bertani
LBDD	Ligand-based drug design
LDH	Lactate dehydrogenase
MALDI-TOF	Matrix-Assisted Laser Desorption Ionisation-Time of Flight
MDL	Molecular Design Limited
$MgCl_2$	Magnesium Chloride
MVV	Medicine for Malaria Ventures
NAD^+	Nicotinamide adenine dinucleotide, oxidized
NADH	Nicotinamide adenine dinucleotide, reduced
NaCl	Natrium Chlorida
NaH_2PO_4	Monosodium phosphate
NCBI	National Center for Biotechnology Information
OXM	Oxamate
QSAR	Quantitative Structure Activity Relationships
PCR	Polymerase chain reaction
PDB	Protein Database
Pf-LDH	*Plasmodium falciparum* lactate dehydrogenase
Pk-LDH	*Plasmodium knowlesi* lactate dehydrogenase
*p*LDH	*Plasmodium* lactate dehydrogenase

Pfcrt	*P. falciparum* chloroquine resistance transporter
*Pf*K13	*P. falciparum* Kelch-13 propeller domain
*Pf*GAPDH	*P. falciparum* glyceraldehyde-3-phosphate dehydrogenase
*Pf*MDR1	*P. falciparum* multidrug resistance 1
PSI	Protein-Specific Iterative
RBC	Red blood cells
RMSD	Root Mean Square Deviation
SAVES	Structure Analysis and Verification Server
SBDD	Structure-based drug design
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
SOPMA	Self-Optimise Prediction Method with Alignment
SP	Sulphadoxine–pyrimethamine
TB	Terrific broth
TCP	Target Candidate Profile
TEMED	Tetramethylethylenediamine
TEST	Toxicity Estimation Software
UFSRAT	Ultra-Fast Shape Recognition with Atom Types
USR	Ultrafast Shape Recognition
USRCAT	Ultra-Fast Shape Recognition with Credo Atom Type
UV-VIS	Ultraviolet–visible spectrophotometry
WHO	World Health Organisation
1,3-BPG	1,3-biphosphoglycerate

CHAPTER ONE

INTRODUCTION

1.1 STUDY BACKGROUND

The World Malaria Report estimates an occurrence of 229 million cases in 2019 over the world (WHO, 2020). In Malaysia, a total of 4630 malaria cases was reported in 2018, in which 4131 cases were mainly caused by *Plasmodium knowlesi* infection and another 499 cases were caused by other Plasmodium species that are known to infect human, such as *P. vivax*, *P. falciparum*, *P. malariae* and *P. ovale* (Chin et al., 2020). Malaysia is located at the equatorial zone with high temperatures and humidity, which is deemed important for transmission of malaria, causing the disease to remain as a public health treat in Malaysia (Jamaiah et al., 2005). In most parts of Peninsular Malaysia, the disease is still under control, but the problem remains persistent in Sabah and Sarawak, where the disease occurred in areas such as in the aboriginal settlements and tribal villages that are mostly found in cleared hilly jungles. Deforestation and associated environmental and population changes have been hypothesized as main drivers of this apparent emergence (Fornace et al., 2016).

There are four species of malaria parasites that are normally associated with human, which are *P.falciparum*, *P. vivax*, *P. malariae* and *P. ovale*. In Malaysia, there is a rise of zoonotic malaria infections among human caused by *P. knowlesi*, which is the species previously known to cause malaria in primates. The evidence of human infection was recorded in the discovery of naturally-acquired *P. knowlesi* infection among people in Kapit Division of Sarawak, Malaysia (Singh et al., 2004). *P. knowlesi* asexual erythrocytic cycle is about 24 hours in human, causing symptoms such as fever that typically occurs at the same frequency and can rapidly develop from febrile illness

into severe malaria, and sometime can lead to death in case of late treatment (Singh et al., 2013) . Severe malaria is life- threatening, due to vital organs dysfunction, for example debilitated lungs, kidneys, liver and brain (Carter & Mendis, 2002). In order to avoid severe illness or casualty, appropriate management of malaria is important.

The management of malaria includes diagnosis and treatment of the disease. The diagnosis of malaria depends upon the summarization of information on clinical observation, previous medical history and laboratory identification of malaria parasites. The treatment of malaria includes drug therapy given to malaria infected patients according to the standard treatment recommended by World Health Organisation (WHO). Artemisinin Combination Therapy (ACT) is the first line treatment for malaria to ease the symptoms, prevent lapse and spread of the disease. Drugs such as chloroquine, malarone, artemether-lumefantrine, mefloquine, quinine and quinidine are also included in treatment of malaria. These drugs are active against the parasites in the blood (CDC, 2015).

Currently, there is no specific drug targeting *P. knowlesi* infection in human. At the moment, the treatment for malaria infection in human that is caused by *P. knowlesi* is built upon drugs that are used to treat malaria infections by other *Plasmodium* species that infect human. Unfortunately, the current drug used is facing major difficulty, due to drug resistance issue, which has been reported in many parts of the world. The emergence of *P. falciparum* resistance towards artemisinin has been reported by WHO in 2013 in five countries near the Great Mekong River such as Cambodia, Laos, Myanmar, Thailand and Vietnam (WHO, 2013).

In Malaysia, there is no report regarding *Plasmodium* resistance towards artemisinin. However, drugs such as mefloquine has been reported to be less sensitive in a study of susceptibility towards antimalarials for malaria patients infected with *P.*

knowlesi, who sought for treatment at the hospitals in Sarawak. It is noteworthy to note that in Malaysia, approximately 70% of malaria cases occurred in Borneo Island, where drug resistance is an emerging problem (Fatih et al., 2013; Jamaiah et al., 2005). Therefore, persistent research for new, non-toxic and functional antimalarial drugs is vital to treat malaria in patients infected with *P. knowlesi.*

One of approaches to solve this issue is by finding substances that are able to target the source of energy for *Plasmodium*, such as glycolysis. *Plasmodium* obtained its energy during the intra-erythrocytic stage in mammalian host for its survival through glycolysis. For this reason, it was postulated that the glycolytic pathway is an ideal target for drug design against *Plasmodium*. In the glycolytic pathway, the final enzyme, which is lactate dehydrogenase (LDH) has been shown to be a potential immune genetic marker besides a good target for chemotherapy (Singh et al., 2012). The fact that *P. falciparum* LDH (*Pf*-LDH) and *P. knowlesi* LDH (*Pk*-LDH) are almost similar in terms of their gene sequences, it is postulated that by targeting that same enzyme in *P. knowlesi* may aid in the treatment of malaria patients and later prevents the spread of the disease.

As the final enzyme in glycolysis, LDH converts pyruvate to lactate, apart from converting NADH to NAD^+ in the final stage of the pathway, hence providing energy for the parasite. Inhibition are defined by the binding affinity in which an inhibitor binds to the free form of the enzyme, or to the enzyme–substrate binary complex or to species that form subsequent to the enzyme–substrate complex along the reaction coordinate of the enzyme, or to both (Buker et al., 2019). Therefore, finding compound that have high binding affinity towards *Pk*-LDH enzyme, could lead to inhibition of the enzyme, hence, lead to parasites cell death It has been proven that compounds that compete with NADH for the active sites of LDH have potentials to inhibit the activity of the enzyme,

thus diminishing the parasites (Penna-Coutinho et al., 2011). Furthermore, the crystal structures of both human LDH and *Pf*-LDH showed two significant variances, which are the location of the NADH factor and the secondary structure of a loop region that closes down on the active site during catalysis (Cameron et al., 2004). No comparison study of human LDH and *Pk*-LDH was performed as the crystal structure of *Pk*-LDH is not yet available. The kinetic differences between human and *Pf*-LDH are very high that the observed LDH activity can be used as an indication of *in vivo* parasitaemia (Alam et al., 2014).

Due to distinctive structural and kinetic properties between human and the malaria parasites' enzymes, targeting LDH enzyme in *P. knowlesi* may aid in the treatment of malaria patients and later prevent the spread of the disease. The challenges deliberated above in fighting malaria due to infection with *P. knowlesi* emphasise the importance of developing novel antimalarial drugs, which may overcome the parasites' resistance issue. The focus of this work is to identify and validate potential compounds that specifically target LDH from *P. knowlesi* (*Pk*-LDH), which can further be developed as antimalarial drugs.

1.2 PROBLEM STATEMENT

Malaria was reported in 97 countries with ongoing malaria transmission. The severity of the disease is exhibited by the number of people developing the symptomatic disease, where in 2019, WHO estimates that 229 million cases occurred around the world and 1819 estimated deaths were recorded in South East Asia (WHO, 2020). According to Management Guideline of Malaria in Malaysia, the incidence rate of malaria in Malaysia over a decade period (1990-2000) ranges from 1.6 to 29.7 per 10,000 person at risk, in a declining trend. The actual number of malaria cases has dropped from nearly

60,000 cases in 1995 to 4725 cases in 2012. Overall, Sabah, Sarawak and Perak states remain stratified as "problematic" malaria areas. The objectives of the National Malaria Control Program are to reduce the national malaria incidence rate to less than 1 per 1,000 population by year 2015 for Peninsular Malaysia and by year 2017 for Sabah and Sarawak. One of the identified strategies to achieve the objective is to provide early case detection and early treatment.

In relation to malaria species, in 2010, *P. vivax* remains the most prevalent parasite species (56%), followed by *P. falciparum* (28%), *P. malariae* (7%) and mixed infections (3%). There is also 6% of malaria species identified as *P. knowlesi*. To date, almost half of malaria cases reported is due to *P. knowlesi* infection. Despite extensive studies in Malaysia on *P. knowlesi* in the 1960s, no additional reports appeared until 2004, when 120 cases of naturally-acquired *P. knowlesi* infection in humans were described in Malaysian Borneo (Singh et al. 2004). It was uncertain whether natural infection of *P. knowlesi* could take place and thus could be a zoonosis. The zoonotic potential of *P. knowlesi* has, until recently, seemed limited, with only sporadic case reports of human infection. Unfortunately, until now, no drugs have been introduced to specifically treat malaria due to *P. knowlesi* (knowlesi malaria) and an effective vaccine is still under development. Hence, drug development against knowlesi malaria is a great priority in managing malaria disease in this country.

Currently, the treatment for knowlesi malaria is based on drugs used to treat malaria caused by other human malaria parasites such as *P. falciparum* and *P. malariae*. Effective treatment of the disease is increasingly compromised by rising resistance of malaria parasites to currently available antimalarials (Turgut-balik et al., 2004). The treatment failure may well occur, and this is mainly due to other factors such as incorrect dosing, inaccuracy on the duration of treatment, misdiagnosis and poor drug quality.