UNIVERSITI PUTRA MALAYSIA

UNRAVELLING THE STRUCTURE OF RIBOFLAVIN SYNTHASE FROM Leptospira kmetyi FOR DESIGNING POTENTIAL ANTI-BACTERIAL DRUG

SAYANGKU NOR ARIATI BT MOHAMAD ARIS

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By

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Thesis Submitted to School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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Chair: Assoc. Prof. Adam Leow Thean Chor, PhD
Faculty: Biotechnology and Biomolecular Sciences

In Malaysia, an increasing number of reported cases of leptospirosis had resulted in significant number of deaths over the past decade. The leptospirosis disease is caused by a bacterial pathogen, *Leptospira* sp. that contaminates soil and water. Like other microorganisms, *Leptospira* sp. have absolute dependency on riboflavin biosynthetic pathway for riboflavin supply. Therefore, targeting this pathway is a potential strategy for treating diseases caused by this kind of pathogen to develop novel and safe antimicrobial agents. In this study, riboflavin synthase from *Leptospira kmetyi*, a local isolate of the pathogen was chosen as a potential drug target as it is an important enzyme catalyzing the last step of riboflavin biosynthesis. To date, there is no crystal structure of riboflavin synthase from *Leptospira* sp. Therefore, the aim of this study is to unravel the crystal structure of the riboflavin synthase from *L. kmetyi* for designing of antibacterial drugs against Gram-negative bacteria. To begin, a computational approach was used in order to identify potential lead compounds that inhibit druggable riboflavin synthase and to determine the stability of the selected inhibitors. Homology modelling was performed using the riboflavin synthase (1I8D) from *E. coli* as a template, where the modelled structure showed a homotrimer of 23 kDa subunits, with three active sites of the trimer identified have to lie a between pair of monomers. In the virtual screening experiment, approximately 1,000,000 commercial drug compounds from the ZINC database were screened to find the best lead. The potential lead compound with the highest docking score (-10.987 Kcal/mol) was identified to be ZINC21883831 which is 2-[(2-chloro-4-fluoro-phenyl) methylsulfanyl]-7-phenyl-3,5-dihydropyrrolo-[2,3-]pyrimidin-4-one. Subsequently, molecular dynamic simulation trajectories were evaluated for 60 ns for stability parameters including root mean square deviation (RMSD), root mean square fluctuation (RMSF), solvent accessible surface area (SASA) and radius of gyration (Rg) of the complexes. Three compounds - ZINC21883831 (Compound1), ZINC66132835 (Compound2) and ZINC38739608 (Compound3) have been found to be highly potential chemicals to serve as inhibitors for riboflavin synthase. While, the objectives in the experimental work are to clone, express, purify, and crystallize the riboflavin synthase in order to determine its 3-

Dimensional structure. Following that, a 612 base pair nucleotide sequence encoding 203 amino acids of riboflavin synthase was cloned into the (pET-22b(+)) vector and expressed in the *E. coli* BL21(DE3) as a His-tag fusion protein. The vector-host combination significantly enhanced the level of the protein expression. The successfully expressed of the His-tag fusion protein was purified using Nickel Sepharose affinity chromatography followed by size exclusion chromatography. Crystals of the purified riboflavin synthase were obtained albeit of poor diffraction quality (4-6 Å). In terms of accuracy, this low resolution data will be detrimental to data interpretation for structure determination. Thus, the focus has been directed towards the improvement of the crystal quality including optimization of the crystallization conditions and manipulation of the grown crystals. A new construct with a thrombin recognition site was designed to remove the flexible C-terminal His-tag. Additionally, ion exchange chromatography was implemented as a polishing step for protein uniformity. The protein was successfully purified and crystallized for X-ray diffraction analysis. Crystal quality obtained from this new construct was improved in terms of its three dimensional shape appearance and quality of diffraction. The *L. kmetyi* riboflavin synthase was solved at 3.2 Å resolution and belonged to the orthorhombic space group $C222_1$ with unit cell parameters a = 59.56, b = 130.63 and c = 202.50 Å. The calculated Matthews coefficient (V_M) was 2.94 $Å^3Da^{-1}$ with a solvent content of 58.24 % and three molecules present in the asymmetric unit. Herein, this study employed computational approach as well as x ray crystallography in order to determine potential inhibitors towards riboflavin synthase. The 3D structural insights gained into riboflavin synthase are excellent as a promising starting points for the rational designs of new effective and safe anti-microbial drugs.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

MERUNGKAI STRUKTUR RIBOFLAVIN SINTASE DARI *Leptospira kmetyi* UNTUK MEREKABENTUK DRUG ANTIBAKTERIA BERPOTENSI

Oleh

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Pengerusi: Prof. Madya. Adam Leow Thean Chor, PhD
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Di Malaysia, peningkatan jumlah kes leptospirosis yang dilaporkan telah mengakibatkan jumlah kematian yang ketara sepanjang dekad yang lalu. Penyakit leptospirosis disebabkan oleh patogen bakteria, *Leptospira* sp. yang mencemarkan tanah dan air. Seperti mikroorganisma lain, *Leptospira* sp. mempunyai kebergantungan mutlak terhadap laluan biosintesis riboflavin untuk bekalan riboflavin. Oleh itu, menyasarkan laluan ini adalah strategi yang berpotensi untuk merawat penyakit yang disebabkan oleh patogen jenis ini untuk membangunkan agen antimikrob yang baru dan selamat. Dalam kajian ini, riboflavin sintase daripada *Leptospira kmetyi*, patogen pencilan tempatan, telah dipilih sebagai sasaran drug yang berpotensi kerana ia merupakan enzim penting yang memangkinkan langkah terakhir biosintesis riboflavin. Sehingga kini, tiada struktur hablur riboflavin sintase daripada *Leptospira* sp.. Oleh itu, matlamat kajian ini adalah untuk merungkai struktur hablur riboflavin sintase daripada *L. kmetyi* untuk mereka bentuk drug antibakteria terhadap bakteria Gram-negatif. Untuk memulakan, pendekatan pengkomputeran telah digunakan untuk mengenal pasti sebatian bahan yang berpotensi yang menghalang riboflavin sintase dan untuk menentukan kestabilan perencat terpilih. Pemodelan homologi dilakukan menggunakan riboflavin sintase (1I8D) daripada *E. coli* sebagai templat, di mana struktur model menunjukkan homotrimer subunit 23 kDa, dengan tiga tapak aktif trimer yang dikenal pasti perlu terletak di antara pasangan monomer. Dalam percubaan saringan maya, kira-kira 1,000,000 sebatian drug komersial daripada pangkalan data ZINC telah disaring untuk mencari bahan terbaik. Kompaun bahan yang berpotensi dengan skor dok tertinggi (-10.987 Kcal / mol) dikenal pasti sebagai ZINC21883831 iaitu 2-[(2-kloro-4-fluoro-phenyl)methylsulfanyl]-7-phenyl-3,5-dihydropyrrolo[2,3-]pyrimidin-4-satu. Selepas itu, trajektori simulasi dinamik molekul telah dinilai untuk 60 ns untuk parameter kestabilan termasuk ralat punca min kuasa dua (RMSD), ralat purata turun naik (RMSF), luas permukaan yang boleh diakses pelarut (SASA) dan jejari lilitan (Rg) kompleks. Tiga sebatian - ZINC21883831 (Kompaun 1), ZINC66132835 (Kompaun 2) dan ZINC38739608 (Kompaun 3) telah didapati sebagai bahan kimia yang sangat berpotensi untuk berfungsi sebagai perencat riboflavin sintase. Manakala, objektif dalam kerja eksperimen adalah untuk pengklonnan, pengekspressan, penulenan dan

penghabluran riboflavin sintase untuk menentukan struktur 3 Dimensinya. Berikutan itu, jujukan nukleotida pasangan bes 612 yang mengekod 203 asid amino riboflavin sintase telah diklon ke dalam vektor (pET-22b(+)) dan diekspresskan dalam *E. coli* BL21(DE3) sebagai protein taupan tag-His. Gabungan vektor-hos meningkatkan tahap ekspresi protein dengan ketara. Protein taupan tag-His yang berjaya diekspresikan telah ditulenkan menggunakan kromatografi affiniti Nikel Sepharose diikuti dengan kromatografi pengecualian saiz. Hablur riboflavin sintase yang telah ditulenkan diperoleh walaupun kualiti pembelauan yang lemah (4-6 Å). Dari segi ketepatan, data resolusi rendah ini akan memudaratkan tafsiran data untuk penentuan struktur. Oleh itu, tumpuan telah diarahkan ke arah peningkatan kualiti hablur termasuk pengoptimuman keadaan penghabluran dan manipulasi pertumbuhan hablur. Konstruk baru dengan tapak pengecaman trombin telah direka bentuk untuk mengalihkan tag His pada terminal C yang fleksibel. Selain itu, kromatografi pertukaran ion telah dilaksanakan sebagai langkah penggilap untuk keseragaman protein. Protein telah berjaya ditulenkan dan dihablurkan untuk analisis pembelauan sinar-X. Kualiti hablur yang diperoleh daripada konstruk baru ini telah dipertingkatkan dari segi penampilan bentuk tiga dimensi dan kualiti pembelauan. Riboflavin sintase *L. kmetyi* telah diselesaikan pada resolusi 3.2 Å dan tergolong dalam kumpulan ruang ortorhombik $C222_1$ dengan parameter sel unit a = 59.56, b = 130.63 dan c = 202.50 Å. Pekali Matthews (VM) yang dikira adalah 2.94 $Å^3Da^{-1}$ dengan kandungan pelarut sebanyak 58.24 % dan tiga molekul hadir dalam unit asimetri. Di sini, kajian ini menggunakan pendekatan pengkomputeran serta kristalografi sinar-x untuk menentukan potensi perencat terhadap riboflavin sintase. Cerapan struktur 3D yang diperoleh ke dalam riboflavin sintase adalah sangat baik sebagai titik permulaan yang menjanjikan untuk rekabentuk rasional drug anti-mikrob baru yang berkesan dan selamat.

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I certify that an Examination Committee has met on 26 August 2021 to conduct the final examination of Sayangku Nor Ariati binti Mohamad Aris on her thesis entitled "Unravelling the structure of riboflavin synthase from *Leptospira kmetyi* for designing potential anti-bacterial drug" in accordance with the Universities and University College Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

Members of the Examination Committee were as follows:

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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This is to certify that:

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- Supervision of responsibilities as slated in Rule 41 in Rule 2003 (Revision 2012-2013) are adhered to.

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Name of Chairman of Supervisory Committee:	Assoc. Prof. Dr. Adam Leow Thean Chor
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bp	Base pair
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Luria-Bertani
LkRS	*L.kmetyi* riboflavin synthase
mLkRS	*L.kmetyi* riboflavin synthase (modified)
NMR	Nuclear Magnetic Resonance
OD	Optical density
PDB	Protein Data Bank
SBDD	Structure Based Drug Design
TBS	Tris buffered saline
TBSTT	Tris buffered saline with Tween 20

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Leptospirosis is a severe neglected zoonotic disease caused by pathogenic Gram-negative bacteria *Leptospira* spp., which can be transmitted directly or indirectly from animals to humans (Paul, 2001; Haake and Levett, 2015; Garba *et al*., 2018). The global impact of this disease is uncertain due to the absence of the data. However, in various parts of the world, leptospirosis is a disease that has a significant health effect and affects people at different socio-economic levels. In temperate countries, the estimated incidence of leptospirosis ranges from 0.1 to 1/100 000 per year to over than 100/100 000 per year during disease outbreaks (Benacer *et al*., 2016). It is estimated that between 300,000 and 500,000 severe cases happen each year where more than 50 per cent will lead to death (Costa *et al.* 2015; Haake and Levett, 2015). In Malaysia, an increasing number of reported cases of leptospirosis had resulted in significant number of deaths over the past decade.

Gram-negative pathogens can infect humans, livestock and marine life. Gram-negative pathogen infection in humans could lead to serious diseases such as tuberculosis, meningitis, and leptospirosis. The inexorable rampage of antibiotic-resistant species has been observed in the last two decades. World Health Organization (WHO) has declared antimicrobial resistance (AMR) as one of the 10 leading global health problems faced by humanity.

In Malaysia, where dengue, malaria and other infectious diseases with conflicting clinical presentations prevail, misdiagnosis of leptospira infection has become a critical issue. This has led to treatment with a wide range of antimicrobials that cover febrile syndromes with various local diseases (Rafizah *et al*., 2012). To avoid complications, efficient and adequate selection of antibiotics is crucial for treatment. Penicillin G, doxycycline, ampicillin or amoxicillin, azithromycin or clarithromycin, and fluoroquinolone, such as ciprofloxacin or levofloxacin, have been used for treatment of leptospirosis. Penicillin G and doxycycline are generally regarded as effective drugs for the treatment of leptospirosis, where doxycycline was commonly used to treat mild leptospirosis and prophylaxis (Rahim *et al*., 2018; Guzman-Perez *et al*., 2021).

Treatment typically follows a route of observational chemotherapy, which requires information on the sensitivity of *Leptospira* species to different antimicrobial drugs. Due to the endemicity of leptospirosis in Malaysia as well as the drastic increase in recorded cases over the last decade, the efficacy of certain antibiotics in controlling this organism is urgently needed (Benacer *et al*., 2013). Benacer *et al.* (2017), reported that, when tested with conventional antimicrobial compounds used for leptospirosis treatment, 65 local *Leptospira* isolates were resistant to trimethoprim and

sulphamethoxazole, as well as a high chloramphenicol minimum inhibitory concentration (MIC).

Naturally occurring genetic changes caused antimicrobial drugs to become ineffective for bacteria, viruses, fungi and parasites, rendering infections life threatening. This emergence of drug resistant bacteria has led to the identification of novel drug target as it is urgently needed to combat this drug resistant pathogen. Referring to WHO (2019) review on antibacterial agents in clinical development, the current clinical pipeline is still inadequate to alleviate the risk of antimicrobial resistance and more innovative products towards pathogens with no cross- or co-resistance to existing groups are desired.

Riboflavin (vitamin B2) is an important component of the basic metabolism. It is a precursor of coenzymes flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) which are vital for multiple cell physiology (Long *et al*., 2010). The Gram-negative bacteria are absolutely reliant on the endogenous biosynthesis of riboflavin for their growth since they are devoid of any flavin uptake system. Furthermore, riboflavin biosynthesis is absent in humans, and they consume riboflavin from their diet. As this biosynthesis is crucial for pathogens but absent in humans, the enzymes involved in riboflavin biosynthesis are regarded as potential anti-bacterial drug targets (Meir and Osherov, 2018).

Recent genomic studies have suggested that the riboflavin biosynthesis pathway is essential for Gram-negative bacteria including Leptospira. Riboflavin synthase is a key enzyme catalyzes the transformation of 6,7-dimethyl-8-ribityllumazine into riboflavin in the last step of the riboflavin biosynthesis pathway. Riboflavin synthase gene is present in the genome of locally isolated virulent *Leptospira* sp., designated as *Leptospira kmetyi* strain sp. nov. (Slack *et. al*., 2009).

Riboflavin synthase structure has been reported in different species such as *Escherichia coli*, *Bacillus subtilis*, *Schizosaccharomyces pombe* and *Brucella abortus*, but none has been reported for *Leptospira* sp. Therefore, the purpose of this study is to unravel the structure of riboflavin synthase in the development of potential anti-bacterial drug. The determination of the 3D structure via X-ray crystallography basically involves six steps which are (i) source purification or cloning, expression and purification of the target macromolecule, (ii) screening for initial crystallization conditions, (iii) optimization of crystal quality, (iv) collection of diffraction data, (v) determination of the structure and refinement of the 3D model and (vi) analysis of the 3D crystal structure. All the procedure and result for those analysis including *in silico* approach for *L. kmetyi* riboflavin synthase are reported in this thesis. Riboflavin synthase from *L. kmetyi* (hereafter referred as LkRS) could be a potential drug target in the development of an antibacterial drug for Gram-negative bacteria, particularly leptospirosis.

1.2 Problem Statement

Leptospirosis can be treated with antibiotics where Penicillin is considered as a first-line therapy for the treatment. The rapid emergence of antibiotic resistant bacteria is a global concern threatening human life. This is particularly worrying in the case of Gram-negative pathogenic bacteria for which effective treatments are limited. Thus, new potent and effective antimicrobial agents are really required. To date, there is no crystal structure available for riboflavin synthase from *Leptospira* sp. which would pave the way for potential antibacterial development, notably in the case of leptospirosis.

1.3 Significance of the study

Unravelling the structure of riboflavin synthase of *Leptospira* sp. could help in the designing of potential drug targetting on key enzyme involves in riboflavin biosynthesis pathway.

1.4 Objectives

The main objective of this research is to unravel the structure of riboflavin synthase from *L. kmetyi*. Unlocking the three-dimensional structure enable us to understand its structure-function relationship as well as to provide a platform for the designing of potential antibacterial drugs. Therefore, this study entails the following objectives:

- To identify potential lead compounds inhibiting druggable riboflavin synthase
- To determine the stability of selected inhibitors towards riboflavin synthase via molecular dynamic simulation
- To elucidate the structure of riboflavin synthase via x-ray crystallography

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