ANTI-PERSISTENT MYCOBACTERIAL INHIBITORS FROM *Streptomyces* sp. H7763 TARGETING ISOCITRATE LYASE AND MALATE SYNTHASE IN THE GLYOXYLATE SHUNT OF *Mycobacterium* sp.

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DECLARATION

I hereby declare that the material in this thesis is my own except for quotations, excerpts, equations, summaries and references, which have been duly acknowledged.

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Ch’ng Ai Ying
20 December 2011
ABSTRACT

ANTI-PERSISTENT MYCOBACTERIAL INHIBITORS FROM *Streptomyces* SP. H7763 TARGETING ISO CITRATE LYASE AND MALATE SYNTHASE IN THE GLYOXYLATE SHUNT OF *Mycobacteria*um SP.

Globally, two billion people are infected with latent tuberculosis (TB) infection, showing asymptomatic immune response but capable to reactivate into chronic TB in life. Immunocompromised patients such as HIV preinfected people have fueled up the reactivation rate while the drug resistant bacilli which evolved without proper medications have resulted in a more complicated treatment. During dormancy, the persistent strain of *Mycobacterium tuberculosis* is non-replicative and recalci
trate to conventional TB drugs which mostly inhibit the biosynthetic cellular process. The persistent strain regulates a switch of metabolism to glyoxylate shunt and uses 2-carbon compounds such as acetate as the primary carbon source to survive. The glyoxylate shunt enzymes namely isocitrate lyase (ICL) and malate synthase (MS) have been structural solved and identified as important virulence and persistence factors. Both ICL and MS thus become attractive targets for anti-persistent TB drug discovery. In the search for persistent TB inhibitors from soil actinomycetes metabolites, a positive extract was obtained and the hits strain was identified and designated as *Streptomyces* SP. H7763. Subsequently, compound P1, P2 and P3 were purified from this butanol extract using bioassay-guided chromatography approaches. P2 was elucidated spectroscopically as 2-amino-3-(cyclohexa-1,4-dienyl) propanoic acid, which was detected for the first time to inhibit *Mycobacterium* sp. As a persistent strain inhibitor, P2 strongly inhibited the acetate grown *Mycobacterium smegmatis* mc²155, H8000 with MIC = 0.02±0.00 µg/mL using a modified resazurin-based microtiter assay (REMA) targeting the glyoxylate shunt. P2 competitively inhibited MS but not ICL against glyoxylate with Ki = 34.85 mM, showing a comparable potency with the control 3-nitropropionate which acted competitively against the acetyl-coenzyme A with Ki = 36.20 mM. Extensive molecular docking studies of P2 with MS was performed and the interaction indicated a better affinity of conformation over the glyoxylate, with a predominant salt bridge bonding between amino group of P2 and Asp 633, a bidentate coordination of the carboxylate group to the Mg²⁺ ion which is essential to the catalytic activity and the cyclohexa-1,4-dienyl ring of P2 that experienced an aromatization inactivation mechanism with Arg 339 which further interrupts the catalytic mechanism. Desiccated P2 was labile to oxidation and aromatised to yield P1, an amino acid which was not bioactive. However, calcium-alginate encapsulated P2 had succeeded to prolong its anti-mycobacterial activity for over 4 weeks and had pre-developed as a slow-release delivery inhibitor to accumulate at the intracellular environment. P2 was not toxic against *Artemia salina* (LC₅₀ > 2000 µg/mL) based on a brine shrimp lethality assay. P3 was a non-selective inhibitor, showing unstable anti-mycobacterial activity against *M. smegmatis* mc²155, H8000 and *M. tuberculosis* H37Rv, ATCC 25618. In conclusion, a persistent *Mycobacterium* sp. Inhibitor P2 targeting the MS was successfully isolated from the *Streptomyces* sp. H7763 culture and may serve as a good lead candidate for further latent TB infection drug design since the enzyme does not exist in mammals.
ABSTRAK

Kini, dua ribu juta populasi dunia telah dijangkiti penyakit tuberkulosis (TIBI) pendam dengan respon imun asimptomatik tetapi akan berakibat menjadi TIBI kronik dalam hayatnnya. Risiko keaktifan TIBI pendam ini bertambah terutamanya pada pesakit yang lemah sistem imun contohnya pesakit pra-jangkitan HIV. Penjangkit baka kebal ubat TIBI pula telah merumitkan perubatan. Semasa berhibernasi, bakteria Mycobacterium tuberculosis baka pendam ini tidak bercampak. Oleh itu, ia adalah kebal terhadap ubat konvensional TIBI yang bersasar pada sistem replikasi sel. Bakteria ini telah menggantikan sistem metabolismenya dengan kitaran glioksilat yang menggunakan sebatian 2-karbon seperti asetat untuk terus berpendam. Enzim kitaran glioksilat iaitu isositrat liase (ICL) dan malat sintase (MS) telah dikenalpasti sebagai faktor pendam dan jangkitan. Maka, kedua-dua enzim ini merupakan sasaran penemuan ubat baru TIBI pendam yang berpotensi. Dalam usaha penemuan perencat TIBI pendam dari ekstrak-ekstrak aktinomisit, satu ekstrak positif telah ditemui dan penghasil metabolit tersebut telah dikena/pasti sebagai Streptomyces sp. H7763. Kemudian, sebatian P1, P2 dan P3 telah ditulenkan daripada ekstrak butanol kultur Streptomyces sp. H7763 tersebut melalui teknik kromatografi berpaduan biosasi. P2 telah dikenalpasti strukturnya sebagai 2-amino-3-(sikloheksa-1,4-diena) propanoik asid secara spektroskopi dan disahkan selanjutnya melalui sintesis. Sebagai perencat baka pendam, P2 telah merencatkan penumbuhan Mycobacterium smegmatis mc²155, H8000 pada sumber karbon asetate dengan MIC 0.02±0.00 µg/mL dalam ujian REMA (resazurin-based microtiter assay) bersasar pada kitaran glioksilat. P2 merencatkan MS dan bakan pada ICL secara berkompetitif dengan glioksilat (Ki = 34.85 mM), aktivitinya setanding dengan kawalan 3-nitropropionat yang merencatkan MS secara berkompetitif dengan asetil-koenzim A. Kajian simulasi pendokan P2 dengan MS telah menunjukkan tarikan affiniti yang lebih baik berbanding dengan glioksilat, terutamanya pada tarakan antara amino P2 dengan Asp 633, satu tarakan 'bidentate' antara karbosilat P2 dengan Mg²⁺ yang penting dalam pemangkinan aktiviti MS dan akhirnya sistem sikloheksa-1,4-diena yang mengalami perencatan secara pengaromatik dengan Arg 339. P2 mudah teroksida dan bertukar strukturnya kepada P1, suatu asid amino yang tidak bioaktif. Sehubungan itu, pengkapsulan P2 dengan kalsium-alginat telah berjaya mengekalkan aktiviti perencatannya lebih daripada 4 minggu dan ia telah dipraperforma untuk berkumpul dalam intrasel supaya lebih bertumpu pada bahagian pendaman. P2 tidak toksik terhadap pertumbuhan Artemia salina (LC₅₀>2000 µg/mL) dalam ujian ketoksikan udang. P3 merupakan sebatian ketiga, menunjukkan aktiviti perencatan yang kurang stabil dan tidak dikenalpasti sasarannya terhadap M. smegmatis mc²155, H8000 dan M. tuberculosis H37Rv, H8000. Kesimpulannya, perencat MS iaitu P2 telah berjaya ditulenkan daripada ekstrak Streptomyces sp. H7763 di mana sebatian ini berpotensi sebagian calon ubat untuk perekaan ubat baru anti-TIBI pendam kerana enzim MS ini tidak wujud dalam mamalia.
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<tr>
<td>3NP</td>
<td>3-nitropropionate</td>
</tr>
<tr>
<td>ACM</td>
<td>Amiclenomycin</td>
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<td>AFCA</td>
<td>(±)-(1S,2R,5S)-5-amino-2-fluorocyclohex-3-ene carboxylic acid</td>
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<td>AMK</td>
<td>Amikacin</td>
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<td>Arg</td>
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<tr>
<td>BCG</td>
<td>Bacillus Calmette-Guérin</td>
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<td>Capreomycin</td>
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<td>CD$_3$OD</td>
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<td>Diode array detector</td>
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<td>DAPA</td>
<td>Diaminopelargnic acid synthase</td>
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<td>DNTB</td>
<td>5,5'-dithio-bis-2-nitrobenzoic acid</td>
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<td>DOTS</td>
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<td>FT-IR</td>
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<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PO</td>
<td>Potassium oxalate</td>
</tr>
<tr>
<td>PRO</td>
<td>Prothionamide</td>
</tr>
<tr>
<td>PYR</td>
<td>Pyruvate</td>
</tr>
<tr>
<td>PZA</td>
<td>Pyrazinamide</td>
</tr>
<tr>
<td>QSAR</td>
<td>Quantitative structure activity relationship</td>
</tr>
<tr>
<td>REMA</td>
<td>Resazurin-based microtiter assay</td>
</tr>
<tr>
<td>RIF</td>
<td>Rifampicin</td>
</tr>
<tr>
<td>RIFAB</td>
<td>Rifabutin</td>
</tr>
<tr>
<td>RIFAZ</td>
<td>Rifalazil</td>
</tr>
<tr>
<td>RIFAP</td>
<td>Rifapentine</td>
</tr>
<tr>
<td>RMSD</td>
<td>Root-mean-square deviation</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>Reverse phase high pressure liquid chromatography</td>
</tr>
<tr>
<td>SAR</td>
<td>Structure activity relationship</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscope</td>
</tr>
<tr>
<td>SPE</td>
<td>Solid phase extraction</td>
</tr>
<tr>
<td>STR</td>
<td>Streptomycin</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris base-acetatic acid-EDTA</td>
</tr>
<tr>
<td>TB</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acids cycle</td>
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<td>TEMA</td>
<td>Tetrazolium bromide microtiter assay</td>
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<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
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<tr>
<td>TNB</td>
<td>2-nitro-5-thiobenzoic acid</td>
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<tr>
<td>TST</td>
<td>Tuberculin skin test</td>
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<tr>
<td>UPLC</td>
<td>Ultra performance liquid chromatography</td>
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<td>World Health Organization</td>
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CHAPTER 1

INTRODUCTION

1.1 Introduction
In 2009, 9.4 million people are infected with tuberculosis (TB) and 1.7 million of them were killed (WHO, 2010). TB is treatable with the discovery of the first antibiotic Streptomycin in 1944 (McKinney, 2000) but the eradication of tubercles still remains an unachievable goal without introduction of more effective medications. Indeed, there have been no novel TB drugs in the market for nearly 50 years; Conventional TB treatment requires an administration of at least six months drugs regimens and 24 months if the tubercles are detected to be drug resistant strains (WHO, 2006a).

Communities with high TB burden are usually world’s poorest people who cannot afford the expensive and protracted medication. Therefore, it is difficult for them to complete the treatments and thereby half million of the impoverished TB patients from China, India, South Africa, Nigeria and Indonesia are infected with multi-drug resistant TB (WHO, 2010). With an over-crowded living condition, this air-borne infectious disease spreads fast within the marginalised communities. Moreover, the exposure of travelers or hospital staff to these communities has increased the infection rate globally and currently two billion people are carrying the tubercles asymptotically as latent TB infection (TB Alliance, 2010).

Patients with latent TB infection are detected positively in tuberculin skin tests or γ-interferon tests but show no clinical symptoms as the tubercles have been arrested by the intracellular activated macrophages in a hypoxic environment (Young et al., 2009). The persistent tubercles realign themselves to increase cell wall thickness and eventually shut off their active replication in order to survive in the oxidative-stressed and nutrient-starved hypoxic environment (Tischler and McKinney, 2010; Russell et al., 2009). Ten percent of these patients will go on to develop active TB in their life and the risk of reactivation is higher if they are
immunocompromised by HIV or chemotherapies. Reactivation of these tuberculosis can lead to chronically active TB which accelerates the spread via blood to all organs in the body.

Conventional TB drugs fail to achieve optimal level within TB lesions against the persistent strains of *Mycobacterium tuberculosis* as the regimens are mainly targeting at the biosynthetic cellular mechanism (WHO, 2006b; TB Alliance, 2008a; Koul et al., 2011). For instance, TB drugs of the aminoglycosides (streptomycin [STR], kanamycin [KAN], amikacin [AMK] or capreomycin [CAP]) inhibit protein synthesis, pyridines (isoniazid [INH], ethionamide [ETA] or prothionamide [PRO]) inhibit cell wall synthesis, quinolones (moxifloxacin [MXF], levofloxacn [LEV] or gatifloxacin [GAT]) inhibit DNA synthesis and ansamycins (rifampicin [RIF]) inhibit RNA synthesis. Therefore, a new inhibitor which has new mode of action or effective against the persistent strains is desired.

When the persistent strains shielding themselves in the sugar limited hypoxic environment, bloated fatty acids (derived from the inflammatory response and dead cells) surrounding thus become their main carbon sources (Ehrt and Schnappinger, 2007). The fatty acids are converted into simpler units such as acetyl-coenzyme A or propionyl-coenzyme A through a $\beta$-oxidation pathway and these two- or three-carbon compounds are then utilised in a glyoxylate shunt that bypass the tricarboxylic acids (TCA) cycle to generate energy. Glyoxylate shunt enzymes of isocitrate lyase (ICL) and malate synthase (MS) are therefore serve as essential drug targets to combat latent TB infection as the persistent strains rely on the glyoxylate shunt to continue to survive within the macrophages.

ICL and MS have been genetically reported to be important virulence factors as the gene-deleted mutant strains have failed to persist in the infected mice (McKinney et al., 2000; Sharma et al., 2000; Muñoz-Elías et al., 2005). The crystal structures of ICL and MS have been fully studied (Sharma et al., 2000; Anstrom and Remington, 2006) and thereby reveal the active site configurations at the atomic level which allow the enzyme-inhibitor interaction to be studied using the computational aided drug discovery tools such as molecular docking and dynamic
simulation. In addition, glyoxylate shunt does not exist in mammalian host as the mammals require sugar as the survival carbon source. Therefore, an ICL or MS inhibitor might be able to minimise the side effects towards the host.

1.2 Rationale of the study
Glaxo Smith Kline (GSK) and TB Alliance have performed an extensive high throughput screening (HTS) to identify ICL and MS inhibitors. From a library of more than one million organic molecules, only a number of leads with phenyl keto butanoic acid (PKBA) backbone have been identified to inhibit MS and currently they are still in the optimisation stage (Freundlich et al., 2010; TB Alliance, 2010). In contrast, the HTS project to search for ICL inhibitors has been discontinued due to the lack of druggability of the ICL targets. Therefore, a more biological variance and diverse scaffolds library is crucial.

Natural product collections have a much higher hit rate than do combinational libraries as it provides novelty and complexity with respect to the number of chirality centers, rings, bridges and functional groups in the molecule. As novel scaffolds are continually needed, natural products and their derivatives remain to be the core resources to obtain novel chemical structures which are active against the ICL or MS.

Bacteria represent the largest source for the natural product drug discovery, followed by plants, fungi and animals (Lawrance, 1999). Among the bacteria, actinomycetes remain to be the most promising novel compound producer as 50% of the reported 22,500 microbe-derived bioactive compounds are actinobacterial members such as Micromonosporineae, Pseudonocardineae, Streptomycineae, Streptosporangineae and some other saprophytic microorganisms (Peláez, 2006; Ashforth et al., 2010). With the fact that top 10 cm of global soil contains $10^{25} - 10^{26}$ actinomycetes, yet only about $10^7$ have been screened for antibiotic production (Baltz, 2007). Therefore, the search of new inhibitors against ICL or MS from these soil organisms is still far from exhaustive.
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