BIOLOGICAL ACTIVITIES OF INHIBITOR OF MALATE SYNTHASE IN THE GLYOXYLATE PATHWAY OF *MYCOBACTERIUM*

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DECLARATION

All the materials in this dissertation are original except for the quotations, excerpts, and summaries and references, which have been duly acknowledged.

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ABSTRACT

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis*. Anti-tuberculosis drugs that are currently available preferentially targeting bacteria during active growth and replication, and requiring long treatment times to successfully clear a TB infection. Glyoxylate shunt or cycle is one of the targets resulting in either growth inhibition or death of the bacteria. Therefore, malate synthase (MLS) in glyoxylate cycle has become a new drug target, particularly for the latent infection. The focus of this study was to isolate new malate synthase inhibitors from a strain of *actinomycetes*, H7763. The fractions were obtained from the bioactive crude acetone extract of H7763 using solvent-solvent extraction and RP-HPLC techniques and tested against malate synthase using (MLS) enzymatic inhibitory assay. The aqueous layer of the extraction of H7763 was found to produce an active fraction (Fraction 7) based on a fractionation condition. The fraction 7 of H7763 was water soluble and gave a partial inhibition zone only in the acetate plate against *M. smegmatis* mc^2155, H8000. The fraction 7 was partially pure as analyzed using RP-HPLC which was assayed for its inhibitory activity using a malate synthase (MLS) enzymatic assay. The results showed a decreased in enzyme and specific activities of H8000 when double volume of the initial concentration of the fraction 7 was added against malate synthase (MLS) of H8000. Therefore, fraction 7 of H7763 can be considered as an enzyme inhibitor of glyoxylate cycle.

AKTIVITI BIOLOGI PERENCAT MALAT SINTASE DALAM KITARAN GLIOKSILAT *MYCOBACTERIUM*

ABSTRAK

Tuberkulosis (TB) merupakan sejenis penyakit berjangkit yang dijangkiti oleh Mycobacterium tuberculosis. *Ubat-ubatan anti-tuberkulosis pada masa kini adalah lebih kepada sasaran terhadap pertumbuhan dan replikasi aktif bakteria tersebut, dan ia memerlukan jangka masa yang panjang untuk rawatannya. Kitaran glioksilat merupakan satu sasaran utama untuk merencat pertumbuhan bakteria tersebut. Maka, malat sintase (MLS) dijadikan salah satu sasaran baru dalam kitaran tersebut untuk melambatkan jangkitan TB. Kajian ini bertujuan untuk memencilkan perencat malat sintase baru daripada sejenis aktinomiset yang diperolehi, iaitu H7763 Ia adalah fokus kepada pemencilan fraksi aktif daripada aktiviti perencatan bagi ekstrak mentah aseton H7763 berdasarkan teknik pemisahan pelarut-pelarut dan kaedah HPLC. Seterusnya, fraksi aktif tersebut diuji dengan ujian enzim. Ekstrak mentah H7763 disediakan serta diekstrakkan dengan aseton. Kemudian, aseton dikeluarkan dan ekstrak kasar tersebut dikeringbekukan. Pemisahan pelarut-pelarut dijalankan terhadap ekstrak kering tersebut sebelum menjalankan pemecahan dengan RP-HPLC. Lapisan akueus hasil daripada pemisahan tersebut menghasilkan satu fraksi aktif (Fraksi 7) pada keadaan pemecahan tertentu dengan RP-HPLC. Fraksi 7 tersebut bersifat mudah larut dalam air dan merencat secara tidak menyeluruh dalam media yang mengandungi asetat sahaja dan bukannya glukosa terhadap M. smegmatis. Separa ketulenan bagi fraksi 7 telah diperolehi selepas ia dianalisi dengan menggunakan RP-HPLC. Dalam ujian enzim, fraksi 7 tersebut didapati merencat dengan lebih berkesan apabila dua kali isipadu daripada kepekatan asal digunakan terhadap malat sintase (MLS) bagi H8000.*

LIST OF SYMBOLS AND ABBREVIATIONS

TB	Tuberculosis
WHO	World Health Organization
DOTS	Directly observed treatment, short-course
HPLC	High performance liquid chromatography
MLS	Malate synthase
MTBC	*Mycobacterium tuberculosis* complex
INH	Isoniazid
PZA	Pyrazinamide
RIF	Rifampicin
EMB	Ethambutol
ICL	Isocitrate lyase
MDR-TB	Multi drugs resistant- tuberculosis
ACS	Acetyl-coenzyme A synthetase
Acetyl-CoA	Acetyl-coenzyme A
TCA	Tricarboxylic cycle
icl	Gene of isocitrate lyase
TFA	Trifluoroacetic acid
^{o}C	Degree of Celsius
psi	psign (pressure)
wv^{-1}	weight per volume
L	Liter
rpm	revolution per minute
gml^{-1}	gram per milliliter
ml	milliliter
µl	microliter
M	mole
mm	millimeter
vv^{-1}	volume per volume
µm	micrometer
%	Percentage
$mlmin^{-1}$	milliliter per minutes

mM	millimole
nm	nanometer
min	minute
U	Units per milliliter of enzyme
Umg^{-1}	Units per milligram of enzyme or protein

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CHAPTER 1

INTRODUCTION

1.1 Background of the study

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis*. It exhibits a penetrance effect in human population that is rivaled by other pathogens (Sharma *et al*., 2000). It claims more human lives each year than any other bacterial pathogen (McKinney *et al*., 2000).

Each year, there are more than 6.5 million new cases of tuberculosis and more than 2 million deaths from tuberculosis worldwide. Tuberculosis is the seventh most important cause of global premature mortality and disability and is projected to remain among the 10 leading causes of disease burden even in the year 2020 (Murray & Salomon, 1998). In 1998, there were an estimated 6.7 million cases of tuberculosis resulting in 2.4 million deaths, making tuberculosis the most lethal infectious disease. It is estimated to exist as a dormant infection in over 2 billion people, awaiting the proper conditions to become activated (Lorenz & Fink, 2002). According to the World Health Organization (WHO), *Mycobacterium tuberculosis* (known as *M. tuberculosis*)

is the major cause of human TB but it can also infect animals which contact with humans (Niemann *et al.*, 2000).

The success of the *Mycobacterium tuberculosis* or bacteria is dependent on it ability to persist and maintain chronic infection in humans. It exists in diverse metabolic states during chronic TB that are not targeted by conventional antimycobacterials (Sharma *et al.*, 2000). Infection is maintained in spite of acquired immunity and resisted eradiation by antimicrobials (McKinney *et al.*, 2000). Zahrt and Deretic (2001) reported that the success of *Mycobacterium tuberculosis* as a pathogen is caused, in part, by its ability to survive in macrophages and establish long term, persistent infection in the host during periods of control by the cell-mediated immunity.

Multiple drug resistant strains of *Mycobacterium tuberculosis*, defined as resistance to at least isoniazid and rifampin, have already caused several fatal outbreaks. This has stimulated a great deal of research aimed at understanding the molecular mechanisms of drug resistance in *Mycobacterium tuberculosis* (Scorpio *et al.*, 1997). The current recommended standard TB chemotherapy, called directly observed treatment short-course (DOTS). It has a cure rates up to 95% and is recommended by the by WHO for treating ever TB patient. Based on the success of DOTS, it is adopted by WHO as a strategy for the global tuberculosis control. However, DOTS alone may not work in areas where there is a high incidence of Multi Drug Resistant-TB (MDR-TB) with the cure rate is as low as 50% (Zhang, 2005). Moreover, WHO also reported that uptake by national program had been slow with

just only 11% of new smear-positive pulmonary tuberculosis cases were enrolled in DOTS programs worldwide (Murray & Salomon, 1998).

Nature produces an amazing variety and number of products. About 100,000 secondary metabolites of molecular weight less than 2500 have been characterized, mainly produced by microbes and plants; some 50,000 are from microorganisms. Secondary metabolism has evolved in nature in response to needs and challenges of the natural environment. Enormous diversity exists in secondary metabolism. Diversity of microorganisms is enormous and only a minor proportion of bacteria and fungi have thus far been cultured and examined for secondary metabolite production (Demain, 1999). Some of their secondary metabolites have employed as useful microbial compounds. Example includes streptomycin from *Streptomyces griseus* for treatment of tuberculosis caused by *Mycobacterium tuberculosis* (Lo *et al.*, 2002).

Sharma (2000) reported that current drugs target only a small number of bacterial processes, such as cell wall formation and chromosomal replication and are under constant threat from emergent drug resistant drain. Despite an urgent need for new therapies targeting persistent bacteria, our knowledge of bacterial metabolism throughout the course of infection remains rudimentary (McKinney *et al.*, 2000). Anti-tuberculosis drugs that are currently available preferentially target bacteria during active growth and replication, requiring long treatment times to successfully clear a TB infection. It is believed that there have drugs which are effective against persistent bacteria will clear an infection more quickly, reducing chemotherapy times and so decreasing the risk of development multi-drug resistant (Smith *et al.*, 2003).

1.2 Objectives

The objectives of the current study were;

a) To isolate active inhibitory fraction(s) from crude extract of H7763 against *Mycobacterium smegmatis* (mc^2155).
b) To determine purity of the active fraction(s) produced by H7763.
c) To determine specific inhibitory activity of the active fraction(s) produced by H7763 using malate synthase (MLS) enzymatic assay.

1.3 Scope of study

This study focused on the biological activities of inhibitors for persistent latent TB infection especially malate synthase (MLS) in the glyoxylate cycle of *Mycobacterium*. *Mycobacterium smegmatis* (*M. smegmatis*) mc^2155 is used as a model for tuberculosis in this study.

The bioactive fraction(s) of the freeze-dried sample of H7763 crude acetone extract was extracted using solvent-solvent extraction as a first phase of purification and then the extract is further fractionated using a Reverse-Phase High Pressure Liquid Chromatography (RP-HPLC) (Hew, 2005). The HPLC bioactive fraction(s) was analyzed using a RP-HPLC to determine purity of the active fraction(s) of H7763. Then, it(s) was evaluated for its biological activities such as inhibitory activity and malate synthase (MLS) enzymatic assay (Dixon & Kornberg, 1959; Sharma *et al.*, 2000). The malate synthase enzyme which was used in this study was from biological

crude extract of *M. smegmatis* mc^2 155, H8000 provided by Dr. John D. McKinney of the The Rockefeller University.

CHAPTER 2

LITERATURE REVIEW

2.1 General features of Tuberculosis (TB)

Tuberculosis (TB) is a major cause of death around the world. This disease is caused by *Mycobacterium tuberculosis* (known as *M. tuberculosis*), a bacterial infectious disease caused by the obligate human pathogen (Glickman & Jacobs, 2001). It is an acid-fast bacillus that is transmitted primarily via respiratory route (Flynn & Chan, 2001). *Mycobacterium tuberculosis* has been classified in genus of *Mycobacterium*, which belongs to the family of *Mycobacteriaceae* of the order of Actinomycetales (Osoba, 2004).

Tuberculosis is a disease that caused by members of *Mycobacterium tuberculosis* complex (MTBC) which comprises the closely relate species *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti*, *M. canetti* (Niemann *et al.*, 2000), *M. bovis*, and *M. caprae* (Skuce & Neill, 2004). The first two are very rare causes of disease and the last two do not cause human disease (Niemann *et al.*, 2000).

It is believed that the members of the MTBC were came from a single successful ancestor based on the genetic homogeneity, with about 0.01%-0.03% synonymous nucleotide variation and no significant trace of genetic exchange among them (Gutierrez *et al.*, 2005).

Mycobacterial species can be divided into 2 groups depend on the growth rate: fast or slow growers (Table 2.1). The definition of slow growth is the bacteria have 0.2 doublings of fewer per hour (generation time more than 5 hours). The fast growers have optimal growth rates of 0.2-1.0 doublings per hour; the slow growers for example *M. tuberculosis*, the growing optimal rate is approximately 0.07 doublings per hour. The slow growers normally produce visible colonies on solid medium within 10-28 days (Colston & Cox, 1999).

From the clinical point of view, mycobacteria are divided into 3 groups based on their pathogenicity, which are:

a. The obligate pathogens (*M. tuberculosis* complex and *M. leprae*).
b. Species that normally live freely in the environment but can cause infections in human. There are 'anonymous', 'atypical', 'tubercoloid', 'non-tuberculous' or 'mycobacteria other than tubercle' (MOTT) bacilli e.g., *M. fortuitum* complex.
c. Species that never cause diseases and are fast-growing mycobacteria e.g., *M. smegmatis* (Collins *et al.*, 1997).

Table 2.1 The 64 species of fast and slow grow *Mycobacterium* (Collins *et al.*, 1997; Gutierrez *et al.*, 2005)

***M. tuberculosis* complex:**			
M. tuberculosis	*M. bovis*	*M. africanum*	*M. microti*
M. pinnipedii	*M. caprae*		
***M. avium* complex (MAC) and related species:**			
M. avium	*M. intracellulare*	*M. lepraemurium*	*M. paratuberculosis*
M. sylvaticum			
Slowly growing photochromogens:			
M. asiaticum	*M. kansasii*	*M. marinum*	*M. simiae*
Slowly growing scotochromogens:			
M. gordonae	*M. scrofulaceum*	*M. szulgai*	
Slowly growing non-chromogens:			
M. branderi	*M. celatum*	*M. gastri*	*M. haemophilum*
M. farcinogenes	*M. malmoense*	*M. nonchromogenicum*	*M. shimoidei*
M. shinshuense	*M. triviale*	*M. terrae*	*M. ulcerans*
M. xenopi			
Rapid growers:			
M. branderi	*M. agri*	*M. aurum*	*M. austroaficanum*
M. chelonae	*M. chitae*	*M. chubuense*	*M. diernhoferi*
M. duvalii	*M. fallax*	*M. flavescens*	*M. fortuitum*
M. gadium	*M. gilvum*	*M. komossense*	*M. neoaurum*
M. obuense	*M. parafortuitum*	*M. phlei*	*M. porcium*
M. pulveris	*M. rhodesiae*	*M. senegalense*	*M. smegmatis*
M. sphagni	*M. thermoresistible*	*M. tokaiense*	*M. vaccae*
Non-cultivable or very poorly growing species:			
M. leprae	*M. genavense*	*M. confluentis*	*M. interjectum*
M. intermedium			

A recognized disease of antiquity, tuberculosis first reached epidemic proportions in the western world during the major periods of urbanization in the 18th and 19th centuries (Ryan & Ray, 1994). It is a disease that is almost exclusively transmitted by aerosolized droplets containing infectious *M. tuberculosis*. These droplets are generated by the cough of a person with *M. tuberculosis* lung

infection and are inhaled by an uninfected person. Thus, *M tuberculosis* is foremost among bacterial pathogen in its ability to establish and maintain latency, a period during which the infected person does not have clinically apparent tuberculosis, but harbours *M. tuberculosis* organisms able to reactivate although the human immune response against it is highly effective in controlling the infection (Glickman & Jacobs, 2001).

Infection occurs in lungs, but the organism can seed any organ via haematogenous spread. Flynn and Chan (2001) reported that there are two possible outcomes for a person encountering *M. tuberculosis* bacilli.

i. It can be immediately destroyed by the host's innate responses. This is a very important area of study for vaccine developments because of the innate mechanisms that protect against infection are largely uncharacterized.
ii. A proportion of persons infected with *M. tuberculosis* develops active tuberculosis within a finite time frame which (1 to 3 years) by the microscopic observation of viable bacteria in their sputum.
iii. It have a clinically latent infection, which there are no symptoms produced that indicate the presence of any tuberculous lesion and it can be achieved through the early restriction of *M. tuberculosis* growth in the lungs before producing TB disease (Gomez & McKinney, 2004).

Infection with *M. tuberculosis* is believed to occur in an alveolar macrophage initially. The bacteria replicate within the macrophage and induce cytokines that initiate the inflammatory response in the lungs. Macrophages and lymphocytes

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