A SUMMARIZED REPORT OF THE THESIS

"SCREENING FOR MICROBIAL INHIBITORS PARTICULARLY AGAINST THE GLYOXYLATE PATHWAY IN Mycobacterium"

THAT WAS SUBMITED TO
THE SCHOOL OF SCIENCE AND TECHNOLOGY
UNIVERSITI MALAYSIA SABAH

FOR THE PROCUREMENT OF
THE DEGREE OF MASTER OF SCIENCE
2003

THIS REPORT IS SUBMITTED TO
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A. Introduction

In the effort to eradicate the modern day Tuberculosis (TB) burden, it is recognized that improvements to the current TB treatment are necessary. This issue has been discussed in great detail in the document entitled "Scientific Blueprint for Tuberculosis Drug Development" (Barry, *et al.*, 2001). Briefly, at least one of the following three improvements needs to be done to the existing TB treatment:

1) To shorten the current 6-8 months drug treatment duration preferably also with significant reduction in the total number of doses to be taken under Directly Observed Treatment, Short Course (DOTS) supervision.
2) To improve the treatment of multi-drug resistant TB, which cannot be treated with isoniazid and rifampicin.
3) To provide more effective treatment of Latent TB Infection (LTBI).

From the chemotherapy point of view, new anti-TB drugs with modes of action different from the current ones are needed. The ideal situation would be to achieve all the above improvements with one new drug.

B. Research Background

1) The ICL gene

The *icl* gene is one of the many *Mycobacterial* genes that have been shown to be up-regulated for their expressions during infection. The product of this gene is isocitrate lyase (ICL), an enzyme of the glyoxylate cycle. This was shown in an analysis, using two-dimensional gel electrophoresis, of the proteins express by *Mycobacterium avium* during intracellular infection of macrophages (Höner Zu Bentrup, *et al.*, 1999). Most interestingly, *Mycobacterium tuberculosis*

with mutation in the *icl* gene has been shown to fail to persist in immuno-competent mice. Furthermore, virulence of this mutant was attenuated (McKinney, *et al.*, 2000). These two studies indicated the requirement of ICL for the intracellular survival of *M. tuberculosis*, and the ability to survive the intracellular environment of macrophage seems to correlate with the ability of the bacilli to persist during the course of infection.

The failure of *icl* mutant *M. tuberculosis* to persist in immuno-competent mice with the concomitant attenuation in virulence makes ICL an attractive drug target. In particular, ICL can be a suitable target for the development of LTBI chemotherapy as the enzyme has been shown to be required for persistence infection at least in mice; and persistent *M. tuberculosis* appeared to be one of the causes of latency in TB infection. In addition, ICL also seems to be absent in animals more highly evolved than worms (Cioni, *et al.*, 1981), and drugs that specifically inhibit ICL should have no side effects in human. This absence makes ICL all the more attractive as a drug target.

2) The ICL screening system

A screening system that screens for inhibitor of ICL has been developed by Sharma *et al.*, (2000) subsequent to the study conducted by McKinney, *et al.*, (2000). The screening system uses the non-pathogenic *Mycobacterium smegmatis* strain mc^2155 (H8000), and the Δ*icl M. smegmatis* mutant (H8001) complemented with the *M. tuberculosis icl* gene as the surrogate target organisms. This screening system is relatively simple, most importantly because the compulsory requirement of the typical Biosaftey Level 3 when working with *M. tuberculosis* is abolished. Briefly, the screening system involves the plating of both the wild type and the complemented *M. smegmatis* on minimal medium agar plates containing either glucose or acetate as the sole carbon source. Discs of filter paper saturated with extracts or ICL inhibitors or antibiotics are placed on the agar plates. In this screening system, inhibitors of ICL are defined by the differential inhibitions observed between the glucose and the acetate minimal

medium plates, in which inhibition zones are present on the acetate plates but absent on the glucose plates.

3) Actinomycetes and myxobacteria

These two groups of bacteria were well-documented for their ability to produce a myriad of bioactive small molecules. The actinomycetes group in particular those in the genus *Streptomyces* have been shown to produce over 500 distinct antibiotic substances (Madigan, *et al.*, 1997). More than 50 of these antibiotics have found practical application in human and veterinary medicine, agriculture, and industry (Madigan, *et al.*, 1997). Other genera of actinomycetes are also known to produce bioactive compounds but their documentation is less extensive in part due to the rarity of these non-*Streptomyces* genera in soil.

The chances of isolating known compounds from actinomycetes, especially from *Streptomyces* are high, as they have been studied extensively for the production of secondary metabolites. However, the study conducted in the other genera of actinomycetes is less extensive and the prospect of finding new compounds from these non-*Streptomyces* actinomycetes may be better. In general, it is hypothesized that novel secondary metabolites are likely to be obtained from novel species of actinomycetes. To increase the chances of isolating novel species of actinomycetes, emphasis was therefore placed on collecting soil samples from different micro-environments.

Studies on the secondary metabolites production in myxobacteria have only been conducted for about twenty years. In comparison, the capability of actinomycetes to produce bioactive compounds had been known since the beginning of the antibiotic era in the 1940s. In the twenty years of intensive studies, particularly at GBF (Gesellschaft für Biotechnologische Forschung) in Germany, myxobacteria have been shown to be a rich source of novel secondary metabolites (Reichenbach and Höfle, 1993). The structures of some of the myxobacterial secondary metabolites had been elucidated but thus far none have

found widespread practical applications. By 1999, only soraphen and epóthilon managed to proceed to the level of industry projects (Reichenbach and Höfle, 1999). The study of secondary metabolites production in myxobacteria is still at its infancy when compared to the studies conducted for actinomycetes. However, the prospect of discovering compounds with novel mode of anti-TB mechanisms from myxobacteria may be brighter as most of the structures of myxobacterial compounds elucidated thus far were new (Reichenbach and Höfle, 1999), and have not been extensively tested for anti-TB properties.

C. Aim of Research

In light of the suitability of ICL as a new target for the development of LTBI chemotherapy and also the availability of a relatively simple screening system, the project aimed to find from soil microbes, a lead compound with inhibitory activity against the mycobacterial ICL by taking advantage of the extensive biodiversity in Sabah. Specifically, secondary metabolites produced by actinomycetes and filamentous fungi isolated from the various ecosystems in Sabah were screened. Although it is undeniably difficult to isolate an active compound from a complex extract mixture such as the bacterial broth culture, Mother Nature however is still the best producer of diverse chemical structures and some of which would have never been synthesized in the laboratory (Duncan and Sacchettini, 2000).

The study also aimed to assess the inhibitory activity of any potential ICL inhibitors obtained from the screening effort, in an *in vitro* cell-free environment. Crude extracts containing the potential ICL inhibitor was assessed enzymatically, using spectrophotometer based on the method developed by Dixon and Kornberg (1959). In this *in vitro* assessment, the ICL of *Bacillus stearothermophilus*, which is available commercially, was used in place of the ICL of *M. tuberculosis*. Ideally, ICL of *M. tuberculosis* should be used but it was unavailable commercially. At present, ICL of *M. tuberculosis* is obtainable from *E.*

coli host transformed with expression plasmid carrying the *M. tuberculosis icl* gene. By conducting this assay, the inhibitory potential of a bioactive compound against *B. stearothermophilus* ICL was assessed and compared to the inhibitory activity of itaconic acid, a known inhibitor of various ICL. For example, itaconic acid has been shown to inhibit the ICL enzymatic activity of *Lupinus* seeds (Vincenzini, *et al.*, 1986) and also of *M. tuberculosis* (Höner Zu Bentrup, *et al.*, 1999). If the bioactive compound potentially inhibits ICL, the same is expected to occur in the enzymatic spectrophotometric assay unless, the inhibitory activity observed from the screening system is not due to the inhibition of ICL or the inhibitory activity is very specific for *M. tuberculosis* ICL. The enzymatic spectrophotometric assay therefore is an attempt to substantiate the ICL inhibitory potential observed in the screening system. In this study, inhibition of the *B. stearothermophillus* ICL is indicated by the decreased in the overall optical densities compared to the standard curve. Optical densities are measured continuously as absorbance at wavelength of 324 nm for 10 – 20 minutes.

D. Materials and Methods

Please refer to **Chapter 2: Materials and Methods** of the thesis.

E. Results

The results obtained from this study can be divided into five parts. Detailed report is made in the thesis. The following is a summary of the results reported in the thesis.

Part 1: Isolation of soil microbes and screening of crude acetone extracts

a) Attempt at isolating myxobacteria was made but turned out unsuccessful.

b) A total of 44 actinomycetes strains were isolated in this study. 7 strains were isolated from 4 soil samples collected from Imbak Valley, and the remaining 40 strains were isolated from 9 soil samples collected from Tabin Conservation Area.

c) A total of 582 different crude acetone extracts were screened in this study. These included crude acetone extracts of actinomycetes isolated from soil collected from the rain forests (115 extracts) and the mangrove ecosystem (113 extracts). 277 of the 582 crude acetone extracts screened were extracts of filamentous fungi; all isolated from soil collected from the rain forests. The remaining extracts were obtained from the aerobic growth of actinomycetes strains. There were 77 extracts that were obtained from the aerobic growth of 40 of the 44 actinomycetes isolated in this study. 37 of the strains were successfully grown in the mannitol-peptone growth medium containing either 1% glucose or 1% sodium acetate. Two different extracts therefore were generated from each of these 36 strains and resulted in a total of 74 crude acetone extracts. Three of the strains, H7793, H7794 and H7800 were successfully grown in the mannitol-peptone medium containing 1% glucose but failed to grow in the same medium containing 1% sodium acetate. The remaining 4 strains, H7774, H7778, H7781 and, H7801 isolated in this study failed to grow in the mannitol-peptone growth medium containing either 1% glucose or 1% sodium acetate.

d) Out of the 582 different crude acetone extracts screened, the actinomycetes strain H7763, obtained from the aerobic growth in the mannitol-peptone medium containing 1% glucose, seemed promising and showed the positive inhibition profile in the ICL screening system. Seven other crude extracts were characterized as toxic: the crude extracts of the actinomycetes strains H7009, H7220, H7358, H8546, H8547, H8549; and the filamentous fungi strain H9009.

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e) Preliminary studies of the crude acetone extract of the actinomycetes strain H7763 indicated that the potential ICL inhibitory entity was:

i) reproducible in independent liquid mannitol-peptone growth medium

ii) stable in terms of the inhibitory activity (inhibitory activity was observable even after more than one year of storage of the extract)

iii) concentration dependent in its inhibitory activity (on the screening plates, the diameters of the inhibitory zones increased with increasing concentration of the active entity tested)

f) Two hypotheses were made and tested based on the ICL screening system. It was an attempt to determine whether the active entity present in the crude extract of the actinomycetes strain H7763 was truly an inhibitor of ICL.

Hypothesis 1: If the active entity was truly an inhibitor of ICL, inhibition zones should be produced on Tween-80 minimal medium plates.
The Outcome: Clearly demarcated inhibition zones were observed.

Hypothesis 2: If the active entity was truly an inhibitor of ICL, the extract should not be toxic to the *icl* mutant *M. smegmatis*.
The Ourcome: "scattered" inhibition zones were observed.

The outcomes from the testing of the above two hypotheses were not able to provide strong evidence for the ICL inhibitory activity of the active compound present in the crude extract of the actinomycetes strain H7763.

g) Two controls were routinely included in the further study of the crude acetone extracts of the actinomycetes strain H7763. The positive control was itaconic acid while the negative control was the crude acetone extract of the actinomycetes strain H7007.

i) Itaconic acid:- In the ICL screening system, itaconic acid produced clearly demarcated inhibition zones on all three types of the minimal medium plates (used in the screening system); the acetate minimal medium plate, the glucose minimal medium plate, and the Tween-80 minimal medium plate. This was observed for the two target organisms used in the screening system (the wild type *M. smegmatis* mc^2155 and the *icl*-transformant). In addition, itaconic acid was also observed to be toxic to the *icl* mutant *M. smegmatis* grown on the glucose minimal medium plate.

ii) Crude acetone extract of the actinomycetes strain H7007:- In the ICL screening system, this extract produced no inhibition zones on all three types of the minimal medium plates. The extract was also non-toxic to the *icl* mutant *M. smegmatis* grown on the glucose minimal medium plates even when tested at high concentrations.

Part II: *In vivo* ICL enzymatic spectrophotometric analysis

This study attempted to determine the ICL inhibitory activity of the crude acetone extract of the actinomycetes strain H7763. The ICL enzyme used was purified from *Bacillus stearothermophilus*, which was purchased from Sigma (catalogue number: I-6754. This *in vitro* cell-free test failed to provide conclusive evidence of the ICL inhibitory potential present in the crude extract of the actinomycetes strain H7763.

Part III: Crude acetone extract of the actinomycetes strain H7372

The actinomycetes strain H7372 was isolated from the mangrove ecosystem. In this study, the crude acetone extract of this strain was observed to produce strong inhibitory activity against the wild type *M. smegmatis* and the *icl*-transformant *M. smegmatis* grown on Tween-80 minimal medium plates. This

was observed when the crude extract was tested at 200 μL. At the same volume of the extract tested, the inhibitory activity on the glucose minimal medium plates and the acetate minimal medium plates, seeded with either the wild type *M. smegmatis* or the *icl*-transformant *M. smegmatis* was extremely weak.

Part IV: Pilot study – using *Escherichia coli* HB101 as the target organism

In this study, the ICL screening system was conducted for the crude acetone extract of the actinomycetes strain H7763, and itaconic acid (serving as control). However, the two target organisms, the wild type *M. smegmatis* and the *icl*-transformant *M. smegmatis* were replaced with *E. coli* HB101 instead. This test was initiated when both itaconic acid, and the crude acetone extract of the actinomycetes strain H7763 produced clearly demarcated inhibition zones on nutrient agar plates seeded with *E. coli* HB101. It was observed from this study that inhibition zone produced by itaconic acid on the acetate minimal medium plate was approximately 4 times larger than the inhibition zone measured on the glucose minimal medium plate. In the case of the crude acetone extract of the actinomycetes strain H7763 (the extract containing the potential ICL inhibitor), the inhibition zone measured on the acetate minimal medium plate was approximately 2 times larger than the inhibition zone on the glucose plate.

Part V: Morphological characterization of strain H7763

On oatmeal agar plates, strain H7763 produced powdery aerial mycelia (the typical morphology of actinomycetes) that were brown in color. Diffusible pigment was not produced but diffusible hydrolytic enzyme may be produced. Light microscopy examination revealed that strain H7763 formed spores in chain that was spiral in shape. In the detection of diaminopimelic acid isomers in the cell wall of strain H7763, using cellulose thin-layer chromatography (TLC), LL-DAP isomers was observed.

F. Discussion

Please refer to the thesis for the detailed scientific discussion.

G. Conclusions

1. At the conclusion of this study, there was no doubt that the crude acetone extract of the actinomycetes strain H7763 contained some form of biological activity. In this study, based on the results obtained from the ICL screening system it was speculated that the extract maybe containing a potential ICL inhibitor. However, several contradictory results were obtained especially in the testing of Hypothesis 2, and also in the *in vitro* ICL enzymatic spectrophotometric assay (this was elaborated and discussed in detail in the thesis). It is of the opinion that the likelihood of the extract to contain an inhibitor of ICL should not be dismissed. This view was expressed based on the comparisons made with the results of the ICL screening system obtained for itaconic acid, which is a known inhibitor of ICL. In the ICL screening system, itaconic acid was observed to produced inhibition zones on all the three types of the screening plates (glucose minimal medium plate, acetate minimal medium plate and Tween-80 minimal medium plate) seeded either with the wild type *M. smegmatis* or the *icl*-transformant. In addition, itaconic acid was also observed to be toxic to the *icl* mutant *M. smegmatis* grown on the glucose minimal medium plate used in the screening system. Therefore, the immediate future research involving this extract will likely include the isolation and purification of the active entity present in the crude extract.

2. The crude acetone extract of the actinomycetes strain H7372 also appeared to be worth for further investigation. In addition to the unusually strong inhibitory activity observed on the Tween-80 plates, this extract may also be containing a potential kinase inhibitor as observed by Ms. Cheah Hwen Yee and

Mr. Lee Kun-Hyung (personal communications). Results and discussion relating to the kinase inhibitory potential of this extract were made in the thesis of Ms. Cheah Hwen Yee, entitled *"Isolation of actinomycetes from mangroves in Sabah and screening for inhibitors against eukaryotic signal transduction"* (submitted to The School of Science and Technology, Universiti Malaysia Sabah, for the procurement of The Degree of Master of Science, 2003)

3. Other possible area for further studies included: a) taxonomic study of the producer strain; the actinomycetes strain H7763 and H7372, and b) the optimization of the ICL screening system using maybe, *E. coli* HB101 as the target organism and that this version of the ICL screening system is tailored specifically for the screening of crude acetone extracts.

H. References

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