SCREENING FOR POTENTIAL INHIBITORS OF *MYCOBACTERIUM TUBERCULOSIS* ISOCITRATE LYASE*:* *IN SILICO* AND *IN VITRO* APPROACHES

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by

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LIST OF ABBREVIATIONS

ADC	Albumin Dextrose Catalase
ADMET	Adsorption, Distribution, Metabolism, Excretion and Toxicity
AIDS	Acquired Immune Deficiency Syndrome
ALA	Alanine
AMBER	Assisted Model Building with Energy Refinement
AR	Analytical Reagent
ARG	Arginine
ASN	Asparagine
ASP	Aspartic Acid
ATCC	American Type Culture Collection
BCG	Bacillus Calmette-Guérin
$CaCl_2$	Calcium Chloride
CHARMM	Chemistry at Harvard Macromolecular Mechanics
CPK	Corey-Pauling-Koltun
CYS	Cysteine
dH_2O	Distilled Water
DMSO	Dimethyl Sulfoxide
Eq	Equation
FF	Force Field
GA	Genetic Algorithm
GB	Generalized Born
GLN	Glutamine
GLU	Glutamic Acid
GLY	Glycine
GROMOS	Groningen Molecular Simulation Computer Program Package
HCS	High Content Screening
HIS	Histidine
HIV	Human Immunodeficiency Virus
HTS	High-throughput Screening

ICL	Isocitrate Lyase
IFU	Instruction For Use
ILE	Isoleucine
LCPO	Linear Combinations of Pairwise Overlaps
LEU	Leucine
LGA	Lamarkien Generic Algorithm
LJ	Lowenstein-Jensen
LYS	Lysine
MBC	Minimum Bactericidal Concentration
MD	Molecular Dynamics
MDR	Multidrug Resistant Strain
MET	Methionine
Mg	Magnesium
$MgSO_4$	Magnesium Sulphate
MIC	Minimum Inhibitory Concentration
MM	Molecular Mechanics
MM-PBSA/GBSA	Molecular Mechanics Poisson-Boltzmann Surface Area/ Generalized Born Surface Area
MMTSB	Multiscale Modeling Tools for Structural Biology
MRC	Multiple Receptor Conformations
MS	Malate synthase
MTB	Mycobacterium tuberculosis
MTP	Micortiter Plates
Na	Sodium
NADI	Natural Product Discovery System
NMR	Nuclear Magnetic Resonance
NPT	Constant Number of Substance, Pressure, Temperature
NVE	Constant number of Substance, Volume, Energy
NVT	Constant number of Substance, Volume, Temperature
OADC	Oleic Albumin Dextrose Catalase
OD	Optical Density
PCA	Principal Component Analysis
PDB	Protein Data Bank

PHE	Phenylalanine
PRMT1	Protein Arginine Methyltransferase 1
PRO	Proline
PTP1B	Protein Tyrosine Phosphate 1B
QM	Quantum Mechanics
RMSD	Root Mean Square Deviation
RMSF	Root Mean Square Fluctuation
SA	Simulated Annealing
SASA	Solvent-Accessible Surface Area
SER	Serine
TAACF	Tuberculosis Antimicrobial Acquisition and Coordinating Facility
TB	Tuberculosis
TCA	Tricarboxylic Acid
TDR	Totally Drug Resistant
THR	Threonine
TIP3PBOX	Transferable Intermolecular Potential Three Point Water Box
TRP	Tryptophan
TYR	Tyrosine
uHTS	Ultra High-Throughput Screening
VAL	Valine
VS	Virtual Screening
WHO	World Health Organization
XDR	Extensively Drug Resistant

LIST OF SYMBOLS

Å	Angstrom
%	Percent
ΔG	Free energy of binding
ΔG_{ELE}	Electrostatic interaction energy
ΔG_{PB}	Polar solvation free energy
µg	Microgram
µl	Microliter
µm	Micro-meter
fs	Femto-second
g	Gram
K	Kelvin
Kcal	Kilo-calorie
L	Liter
logP	Logarithm of the partition coefficient
M	Molar
mg	Milligram
ml	Milliliter
mm	Millimeter
nm	Nano-meter
ns	Nano-second
^{o}	Degree
^{o}C	Degree Celsius
ps	Pico-second
rpm	Revolution per minute
α	Alpha
β	Beta

PENYARINGAN PERENCAT BERPOTENSI UNTUK PERENCATAN ISOCITRATE LYASE *MYCOBACTERIUM TUBERCULOSIS*: PENDEKATAN *IN SILICO* DAN *IN VITRO*

ABSTRAK

Tuberkulosis (juga dikenali sebagai penyakit batuk kering atau penyakit tibi) merupakan sejenis penyakit yang dijangkit oleh bakteria *Mycobacterium tuberculosis* (MTB). Wabak global ini telah menjangkiti satu per tiga populasi dunia tanpa mengira jenis jangkitan aktif atau laten. Dalam dua jenis jangkitan ini, MTB menjalani metabolism yang berbeza. Contohnya mereka mempunyai kitaran janaan tenaga yang berbeza semasa aktif dan laten. Bukannya menyasarkan sasaran ubat yang terlibat dalam metabolism semasa MTB aktif, penyelidikan ini menyasarkan isocitrate lyase (ICL), satu sasaran ubat yang terlibat dalam kitaran janaan tenaga semasa MTB dalam keadaan dorman (juga dikenali sebagain glyoxylate cycle). Kajian sebelum ini menunjukkan bahawa MTB yang dorman tidak dapat hidup dalam paru-paru model tikus tanpa ICL. Pada masa yang sama, ICL tidak wujud dalam mamalia. Jadi, ICL adalah sesuai digunakan sebagai sasaran ubat. Dalam kajian ini, simulasi dinamik molekul (MD, molecular simulation) dilakukan ke atas struktur kristal ICL terlebih dahulu dengan pakej AMBER 8. Hasil daripada 30 ns simulasi MD telah menunjukan bahawa lingkaran C-termina terlibat dalan jalan masuk ke tapak aktif. Melalui pengiraan MM-PBSA, didapati bahawa pengikatan substrat (isocitrate) adalah disumbangi oleh interaksi electrostatik dan hidrofobik. Kesatuan konformasi ICL yang dikumpul melalui simulasi MD telah digunakan untuk menyaring 3,000 kompoun kimia daripada tumbuhan tempatan Malaysia (NADI database). Penyaringan maya ini dilakukan dengan program AutoDock 3.05. Adalah didapati bahawa 22 calon

berpotensi telah memenuhi syarat Lipinski's Rule of Five (kriteria ubat-rupa). Dalam 22 calon berpotensi, 6 boleh didapati secara komersil sebagai kompoun tulen malah 16 yang lain hanya boleh didapati melalui 12 tumbuhan (akhirnya hanya 10 tumbuhan sahaja yang didapati). Jadi 6 kompoun tulen dan 10 ekstrak tumbuhan telah digunakan dalan ujian aktiviti perencatan iaitu ujian enzim dan ujian disk difusi. Ujian enzim dilakukan dengan mengguna MTB ICL yang diklon, diekspres dan ditulen malah ujian disk difusi mengguna model gantian MTB, iaitu *M. smegmatis* untuk menguji perencat ICL yang berpotensi cadangan penyaringan maya. Namun itaconate, perencet ICL yang diketahui dapat menunjuk aktiviti perenjatan, tetapi kompoun tulen dan ekstrak tumbuhan tidak. Dalam ujian disk difusi, empat ekstrak telah menunjukkan perenjatan aktiviti ICL iaitu ekstrak *Manilkara zapota, Momordica charantia, Vitex negundo* dan *Morinda citrifolia*, dengan kepekatan renjatan minima (MIC, minimum inhibitory concentration) masing-masing sebanyak 12.5 mg/ml, 12 mg/ml, 0.78 mg/ml dan 0.39 mg/ml. Kepekatan baktericida minima *M. zapota* dan *M. charantia* adalah 25 mg/ml. Malah kepekatan baktericida minima *V. negundo* dan *M. citrifolia* adalah 1.6 mg/ml. Kombinasi eksperimen *in silico* dan *in vitro* ini telah berjaya mengenal pasti perencat MTB ICL yang berpotensi. Akan tetapi, pengoptimuman juga diperlukan untuk siasatan lanjut, contohnya pengajian aktiviti perencatan dengan ekstrak pecahan, pengenalan pasti kompoun yang terlibat dalan perencatan dan ujian lanjut atas kepekatan ekstrak yang berbeza dalam ujian enzim.

SCREENING FOR POTENTIAL INHIBITORS OF *MYCOBACTERIUM TUBERCULOSIS* ISOCITRATE LYASE*:* *IN SILICO* AND *IN VITRO* APPROACHES

ABSTRACT

Tuberculosis (TB) that is caused by *Mycobacterium tuberculosis* (MTB) still remains as global epidemic and affects one-third of the world population, regardless of active TB or latent TB infection. The metabolism for active and latent MTB are different, for instance they have different energy generation cycle during active and latent stage. Instead of focusing on the drug targets during the active stage, which already have the effective therapeutic drugs, this study targeted isocitrate lyase (ICL), a potential target for dormant (latent) MTB energy generation cycle (glyoxylate cycle). Studies showed that without ICL, dormant MTB cannot survives within the lung of a murine model. Besides, this enzyme is not found in mammals hence it is an appropriate drug target to eliminate TB. In this study, molecular dynamics (MD) simulations were first performed on the crystal structure of ICL, using AMBER 8 molecular dynamics package. Results from 30 ns of MD simulations showed that C-terminal loop is involved in the access towards the active site. In addition, MM-PBSA calculation showed that the substrate (isocitrate) binding was contributed by electrostatic interaction as well as the hydrophobic interactions. Ensemble conformations obtained from MD simulation were then used for virtual screening to search for the potential ICL inhibitor from Malaysian local plant database (NADI) with the collection of 3,000 compounds. Virtual screening that performed with AutoDock 3.05 has managed to identify 22 potential candidates which are within Lipinski's Rule of Five (with drug-likeliness criteria). Of the 22 candidates, 6 were commercially available as pure

compound while the remaining 16 candidates can only be obtained from 12 plants (however, only 10 plants were available during the study). Therefore, 6 pure compounds and 10 crude extracts were used to test for their inhibition activity by enzymatic assay and disc diffusion assay. In enzymatic inhibitory assay, MTB ICL was cloned, expressed and purified whereas disc diffusion assay used a replacing MTB model, *M. smegmatis* to test against the potential inhibitors suggested from virtual screening. The known inhibitors of ICL, itaconate managed to show inhibition in enzyme inhibitory assay but the pure compounds and crude extracts were not. Astonishingly, four crude extracts: *Manilkara zapota*, *Momordica charantia*, *Vitex negundo* and *Morinda citrifolia* showed inhibitory effect on *M. smegmatis* with minimal inhibitory concentration (MIC) of 12.5 mg/ml, 12 mg/ml, 0.78 mg/ml and 0.39 mg/ml, respectively. *M. zapota* and *M. charantia* obtained minimal bactericidal concentration (MBC) of 25 mg/ml whereas *V. negundo* and *M. citrifolia* have MBC of 1.6 mg/ml. The combination of both *in silico* and *in vitro* approaches managed to identify potential compounds for MTB ICL. However, optimization will be needed in future study e.g. inhibition study on the fractionated crude extract, identification of compounds that gave inhibition effects and further testing on different concentration of crude extract for enzymatic assay.

CHAPTER ONE

GENERAL OVERVIEW

1.1 Statement of the problem

It is indisputable that *Mycobacterium tuberculosis* (MTB) is a resurgent infectious disease. Compounded to the problem are the multidrug resistant, extensively drug resistant strains and totally drug resistant strains. The development of current potential drugs, which mainly target on active phase MTB, is bound to face a certain extent of challenges when the drugs are no longer effective against the targets. The research on new drug target and new source of drug has thus become tougher and time consuming. This could be one of the reasons for no new treatment regime available since 40 years ago. In addition, no drug has been developed to target on latent phase MTB. If new drug can target on dormant phase MTB, TB dormancy might be able to be eradicated.

1.2 General objective of the study

The initial part of this study was to investigate the dynamics of MTB ICL and to screen against Malaysia Nature Product database (NADI) for MTB ICL potential inhibitors. The potential inhibitors from the screening was then is to test with *in vitro* experiment to evaluate their inhibitory potential by *in vitro* experiment.

1.3 Tuberculosis

Tuberculosis (TB) is a lethal infectious disease caused by *Mycobacterium* spp., specifically *Mycobacterium tuberculosis* (MTB), first discovered in 1882 by Robert Koch (Youmans, 1979). MTB spread through air when an infected individual coughs, sneezes and talks. A sneeze with 3,000 nasal droplets would release 10,000 active MTB to the air. (Rinaggio, 2003). These MTB can float in the air by attaching to the dust and remain active in the air for several hours (Russell *et al.*, 2010). Upon infection, these strict aerobic and acid-fast MTB can form tubercles at infected area and cause cellular necrosis, hence tuberculosis (Youmans, 1979).

According to World Health Organization (WHO) Tuberculosis Facts (latest updated in year 2014), one-third of the world population has been infected with TB. A total of 95% TB mortality cases occurred in the developing countries. In Malaysia, Malaysian Health Fact from Ministry of Health Malaysia reported that TB has the highest mortality rate among the infectious diseases. Despite the availability of drugs, TB therapy has been challenged by the long treatment regime which is last from 6 to 24 months, multidrug resistant strains, HIV/AIDS and poverty. Under the premise of no new drug available in the past 40 years, this situation has spurred the urgent search for novel TB drug and its target.

MTB survives in two phases, the replication phase (active TB) and the dormant phase (inactive TB). In these two different phases, MTB utilizes different metabolism pathway for different energy source. MTB consumes the carbohydrate and lipid as sole carbon source in replication and dormant phase, respectively. As these two phases undergo different metabolism pathways, possibility to share common enzyme is rather low. Under this circumstances, to select a common enzyme as the drug target which is

simultaneously important for both survival phase of MTB could be a pipe dream. Current available drugs are targeting at the enzyme involved in replication phase of MTB only, including one of the latest potential drug – TMC207. TMC207 which is currently in phase 2 efficacy study surprisingly showed bactericidal property against dormant MTB (Koul *et al.*, 2008). However, no drug was available for those in dormant phase at the moment.

1.3.1 Tuberculosis infection

MTB can trigger primary infection (acute infection; MTB in replication phase) and also latent infection (persistent infection; MTB in dormant phase). Primary infection can be handled by cell-mediated immune response when MTB starts to replicate (Rinaggio, 2003). Although MTB are slow growing, they are still able to replicate before eliminated by slower cell-mediated immune response. When a patient is infected by MTB, MTB will usually attacks lung cavity to develop pulmonary TB as MTB will be phagocyted by alveolar macrophage when they enter via airways (Mohan, 2004; Russell *et al.*, 2010). As shown in Figure 1.1, when MTB-phagocyted macrophage forms granuloma, the granuloma will undergo differentiation to become a more vascularised structure to mineralize and sterilize the infection (Russell *et al.*, 2010). Otherwise, the granuloma will undergo necrosis under certain circumstances such as HIV-infected or malnutrition, to release the infectious mycobacteria back to the airway (Russell *et al.*, 2009).

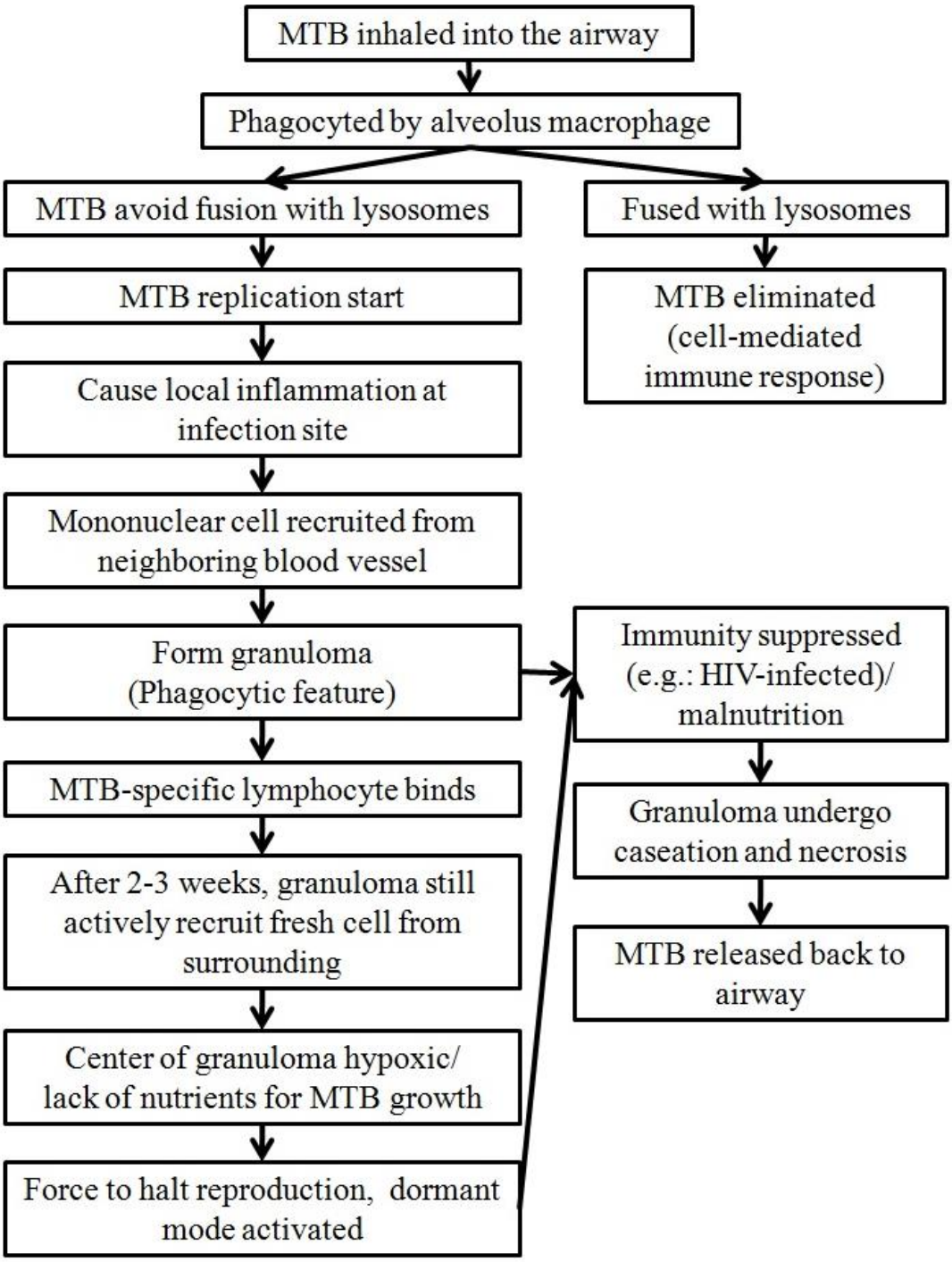


Figure 1.1: Summary of *Mycobacterium tuberculosis* life cycle in human host.

Other than pulmonary TB, extra-pulmonary TB might also occur such as the central nervous system, the lymphatic system, the circulatory system, the genitourinary system, the gastrointestinal system, bones, joints, and even the skin (Mohan, 2004). Other species of *Mycobacterium* such as *Mycobacterium bovis*, *Mycobacterium africanum*, *Mycobacterium canetti*, and *Mycobacterium microti* can also cause TB but they are less common in human (Niobe-Eyangoh *et al.*, 2003). TB patients usually get fatigue easily, losing weight, having fever and night sweat. In more serious cases, symptoms such as coughing, chest pain, coughing out blood-containing sputum, and difficulty in breathing will be observed (Arora, 2009). Further diagnose with chest x-rays, analysis of sputum, and skin tests can be performed to confirm the disease. TB vaccine, Bacillus Calmette-Guérin (BCG) is available and there are also therapeutic TB drugs. To date, tuberculosis had killed more than 1.6 million mankind annually and persisted as latent infection in one-third of the world population (Ranjeet and Vinod, 2008; W.H.O, 2016). As MTB are able to cause persistent infection in immuno-suppressed patients, HIV/AIDS population is one of the high risk populations.

1.3.2 Drug resistance of *Mycobacterium tuberculosis*

The therapeutic drugs are divided into first-line drugs and second-line drugs. The first-line drugs are isoniazid, rifampin, ethambitol and pyrazinamide whereas the second-line drugs include streptomycin, kanamycin, ethionamide, para-aminosalicyclic acid, ofloxacin, capreomycin, amikacin and cycloserine (Zumla *et al.*, 2013). However, the thick and waxy cell wall of MTB that is rich in novel lipids and polysaccharides formed a challenging barrier for the drugs to reach the respective target. Therefore, the treatment process is no doubt to be lengthy (Baker, 2007). During first six-months treatment using the combination of first-line drugs, MTB will

have sufficient time to establish drug resistance, even though they are extremely slow growing bacteria with doubling time of about 24 hours (Baker, 2007). If the patient could not be cured within the first 6 months, prolonged treatment up to two years with the second-line drug is needed to combat the drug resistant strain (Zumla *et al.*, 2013).

MTB mostly establishes drug resistance through chromosomal mutation (Johnson *et al.*, 2006). When the attack of drugs cause the drug target's gene mutation, the mutated strain (drug resistant strain) will keep replicating with the chromosome. Fortunately, the chromosomal locus that used to resist each drug is different (Johnson *et al.*, 2006). Theoretically, the possibility for wild type MTB to obtain mutation more than two chromosomal loci simultaneously (multidrug resistant strain, MDR) is rather low. Yet, due to the low awareness, the MDR strains manage to be spread. The MDR MTB strain is define as MTB which is resistant to at least both first line drug of isoniazid and rifampin (Johnson *et al.*, 2006). Nowadays, not only MDR strain is getting more prevalence, extensively drug-resistant (XDR) strain that required third line drug (Zumla *et al.*, 2013) and totally drug-resistant (TDR) strain that resist to all first and second line drug is emerging fast (Velayati *et al.*, 2009; Rowland, 2012; Udwadia *et al.*, 2012).

The current available drugs has low efficacy towards MTB in dormancy phase because they target only the drug targets involve replication phase (Getahun *et al.*, 2015). If there is any drug available for dormant MTB in the future, another challenge that can be foreseen is the drug delivery to dormant MTB-containing macrophage which is difficult to identify. As the drug during the dormant phase is still unavailable, the problem of drug resistance during the dormant phase is still unknown.

1.3.3 *Mycobacterum tuberculosis* in dormancy

Replicating MTB located at the macrophage-rich centre of granuloma. When MTB is replicating, granuloma will actively recruits cell. Meanwhile, it is being surrounded by MTB-specific lymphocytes and enclosed by fibrous cuff. When granuloma increases in size, oxygen and nutrients could be hard to transport into the macrophage-rich centre (Russell *et al.*, 2010). It is believed that when the macrophage-rich centre of granuloma become hypoxic or nutrient deprivation, MTB will enter the dormant phase (Gengenbacher *et al.*, 2010). However, it is not an easy task to study the *in vivo* macrophage environment. No work has successfully obtained an *in vivo* model of dormant MTB, only relied on several *in vitro* models. There are two well known *in vitro* models which study dormant MTB with different environment: the Wayne's model (Wayne and Hayes, 1996) and Loebel's model (Betts *et al.*, 2002). Wayne's model studied the dormant MTB behaviour in hypoxic yet nutrient rich medium but Loebel's model used nutrient deprivation yet oxygen rich medium. Although both models have verified the viability of dormant MTB in respective survival environment, no data could actually confirm on which model can represent the real macrophage environment (Gengenbacher *et al.*, 2010).

Dormant phase MTB will be in non-replicating mode. In order to survive within the unfavourable environment in macrophage, MTB shifts its metabolism pathway. One of the obvious change is the carbon source during nutrients deprivation (Betts *et al.*, 2002). Dormant MTB utilizes fatty acid or lipid as their sole carbon source instead of carbohydrate. The energy of MTB is produced via glyoxylate bypass instead of oxygen- dependent tricarboxylic acid cycle (TCA cycle; also known as Krebs cycle) (Singh and Ghosh, 2006). This interesting finding has lead the dormant MTB research to another milestone which is related to glyoxylate bypass.

1.3.4 Isocitrate lyase as drug target

Krebs cycle is a common energy generating pathway if carbohydrate is the main carbon source. However, under nutrient depleted condition, global gene expression of MTB would shut down, including the gene expression for Krebs cycle (Betts *et al.*, 2002). However, gene expression for glyoxylate pathway would not be affected (Gengenbacher *et al.*, 2010). Glyoxylate pathway is a bypass of Krebs cycle or sometimes called the modified Krebs cycle (Dunn *et al.*, 2009). Without ordinary substrate of carbohydrate, nutrient-starved dormant MTB utilizes lipid as the sole carbon source to generate energy.

Figure 1.2 shows the comparison of Krebs cycle and glyoxylate bypass. The early phase of glyoxylate pathway is similar to Krebs cycle. Acetyl-CoA is the only substrate for both glyoxylate and Krebs cycle but the source of acetyl-CoA is different. Carbohydrate has to undergo glycolysis to generate acetyl-CoA for Krebs cycle. Meanwhile, lipid has to undergo beta-oxidation to obtain acetyl-CoA for glyoxylate cycle. The point of divert for these two pathways begin when acetyl-CoA converts into isocitrate. For the glyoxylate pathway, two important enzymes are needed namely isocitrate lyase (ICL) and malate synthase (MS). Isocitrate lyase is responsible to cleave the isocitrate into glyoxylate and succinate whereas malate synthase converts glyoxylate into malate by adding an acetyl group.

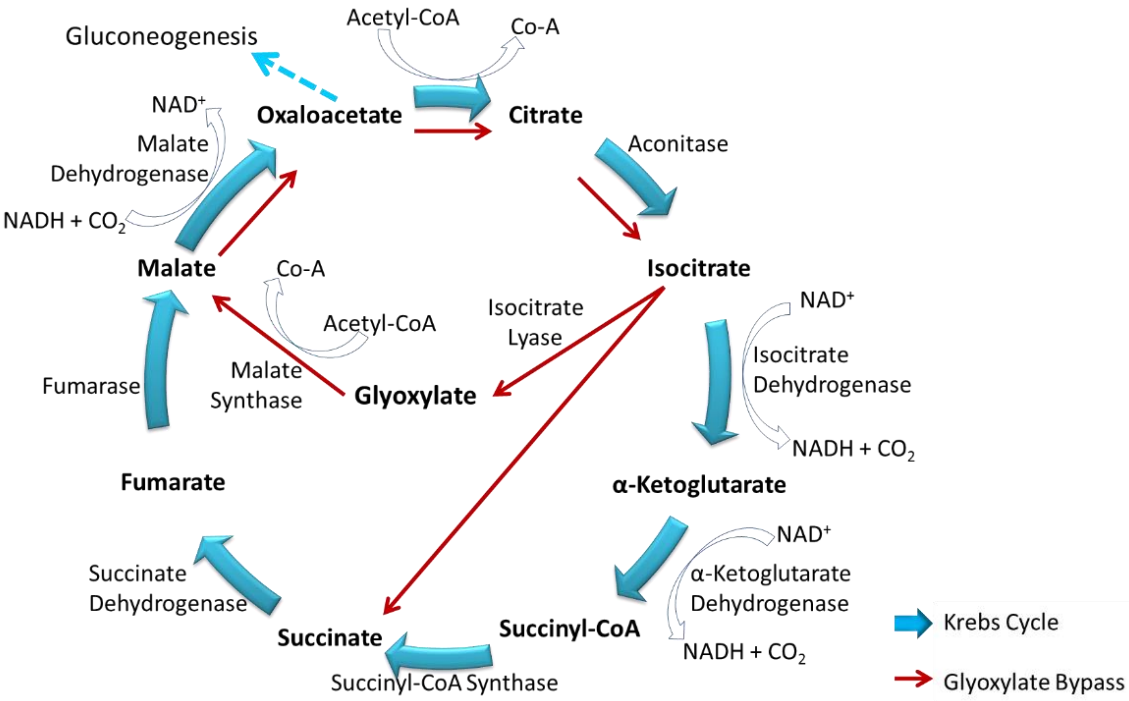


Figure 1.2: The Krebs cycle and the glyoxylate pathway for energy generation. The blue bold arrow indicates the Krebs cycle and the red arrow shows the glyoxylate bypass.

Both ICL and MS were potential drug targets. The drug ability of MS has been proved and various studies have been carried out to optimize its usage (Smith *et al.*, 2003; Kinhikar *et al.*, 2006; Arora, 2009; Krieger *et al.*, 2012). On the other hand, several high throughput screening has been carried out to search for ICL inhibitor at the same time. It was found that its drug availability is not as good as MS. One explanation found regarding the cavity size of the active site. ICL active site is relatively small in capacity compared with that of MS (Krieger *et al.*, 2012; Sharma *et al.*, 2000). Other than this, not much research has been published to further explain about the low drug availability of ICL. This has incited the interest in this study to understand ICL.

1.3.5 Isocitrate lyase related studies

The structure of MTB ICL in Figure 1.3 was solved in year 2000 by Sharma *et al.* They reported that ICL is stable as dimers but functional in tetramer. Each of the subunits has an unusual α/β barrel as the largest core domain which is formed by of eight α-helix and β-strand respectively. Each subunit has an extra helix that projected out from the barrel and another two ensuing helix involved in the interaction among the subunits. On top of the barrel, there is an important small β-domain which has several active side residues.

There are a total of three ICL structures solved by Sharma *et al.*(2000). The first structure is a ligand-free ICL which is in “open conformation” (PDB id: 1F61). When ligand glyoxylate and succinate (PDB id: 1F8I); or pyruvate (PDB id: 1F8M) binds, they trigger a conformational change to make the ICL shift into a “close conformation”. ICL is an enzyme that is able to perform reversible catalysis of

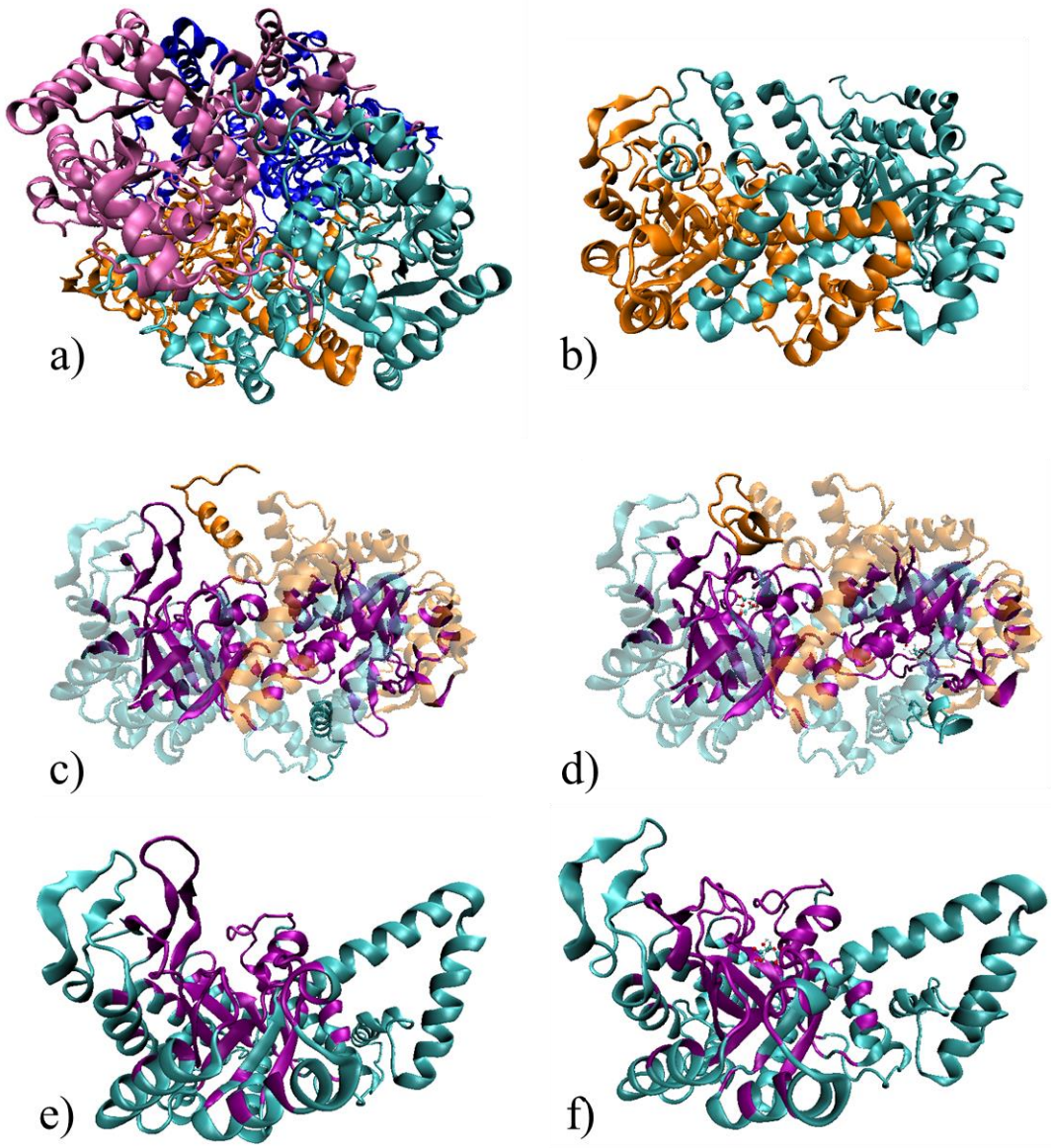


Figure 1.3: The structure of ICL from MTB. a) Tetramer, each colour represents a subunit of ICL; b) dimer, cyan and orange represents monomer 1 and monomer 2; c) dimer in open conformation; d) ICL dimer in close conformation; e) monomer 1 in open conformation without ligand; and e) monomer 1 in close conformation with ligand. Purple represents the active site (Sharma *et al.*, 2000; Lee *et al.*, 2015).

isocitrate, meaning that it is able to cleave as well as form isocitrate. However, no study has been published on the interaction between the ICL and isocitrate. The catalytic mechanism of forming isocitrate form glyoxylate and succinate was only partly described (Sharma *et al.*, 2000). The cleavage mechanism of isocitrate to produce glyoxylate and succinate is remain unknown.

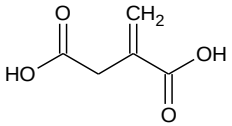
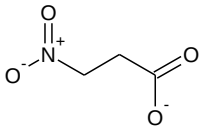
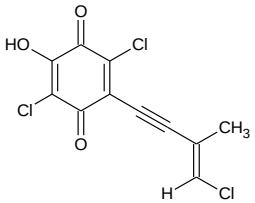
According to Dunn *et al.* (2009), ICL gene is not found in any placental mammals. Muñoz-Elías and McKinney (2005), showed that there are 2 types of ICL exist in MTB: the ICL 1 (prokaryotic-like isoform) and ICL 2 (eukaryotic-like isoform). ICL1 and ICL2 are jointly required for MTB. Absence of either one of them causes little effect for MTB but absence of both will cause MTB being eliminated from the lungs. Gould *et al.*(2006) reported that ICL 1 has dual roles. ICL 1 is needed in both glyoxylate cycle and methylcitrate cycle in MTB. Methylcitrate cycle is a cycle that removes the by-product of lipid beta-oxidation, the propionyl-CoA, which is toxic to MTB. In this cycle, 3 enzymes needed are methylcitrate synthase, methylcitrate dehydrogenase and 2-methylisocitrate lyase (MCL). It was found that MTB only produces methylcitrate synthase and methylcitrate dehydrogenase but not MCL. The function of MCL was carried out by ICL 1. This study has thus shown that ICL is more important than expected (Gould *et al.*, 2006).

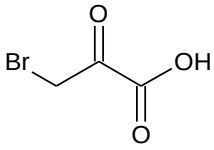
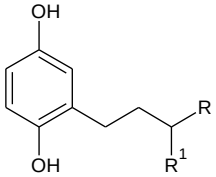
Another research topic highlighted is the screening of inhibitor of ICL. According to the review by Dunn *et al.*(2009), the well-known ICL inhibitors include itaconate, 3-nitropropionate, 3-bromopyruvate, malate and oxalate (Bentrup *et al.*, 1999; McFadden and Purohit, 1977). Most of the inhibitors were analog of the natural substrate. However, they were later proven toxic and non-specific to develop into drug as they will inhibit some of the important metabolism enzymes which are simultaneously exist in both human and MTB. For instance, one of the succinate

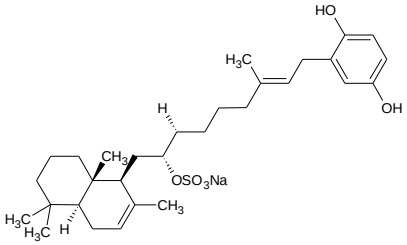
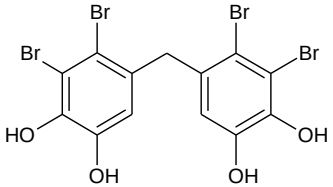
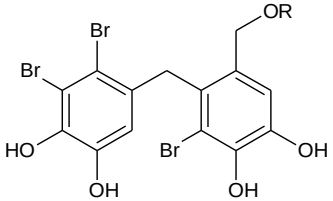
analogs, 3-nitropropionate cause neurotoxicity (Greene *et al.*, 1998). Another succinate analog, itaconate affect the growth of rat (Booth *et al.*, 1952) and also cause hypertonicity for cat's blood pressure (Finkelstein *et al.*, 1947). Whereas, the analog of glyoxylate, 3-bromopyruvate is an energy blocker (Ko *et al.*, 2004; Shoshan, 2012).

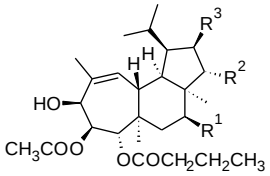
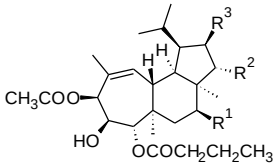
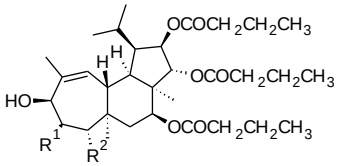
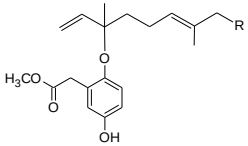
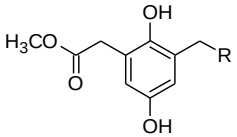
There are a few high throughput screening of ICL were reported. Two groups have reported their works in year 2006. First project is carried out by Global Alliance for TB Drug Development and GlaxoSmithKline, extensively screen for ICL inhibitor via high throughput screening. However, the project was terminated as the outcome was modestly successful (Global Alliance For TB Drug Development, 2010) In the same year, a Chinese group searched for potential ICL inhibitors from traditional Chinese medicine. They concluded that extracts form *Illicium verum* and *Zingiber officinale* were able to inhibit ICL (Bai *et al.*, 2006). Unfortunately, the lead compound for ICL inhibition is remains unknown. In later years, the searching efforts continued by some groups with their respective in house chemical compound library. Their HTS managed to identify lead compound I2906 (Lu *et al.*, 2010), Ydcm67 (Ji *et al.*, 2011) and IMBI-3 (Liu *et al.*, 2016) as the potential inhibitor for ICL. More ICL potential inhibitors that was screened with various strategies such as using MTB ICL and non-MTB ICL as well as screening from natural compound or synthetic compound were summarized in a Table 1.1 (Lee *et al.*, 2015).

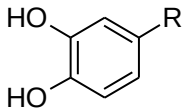
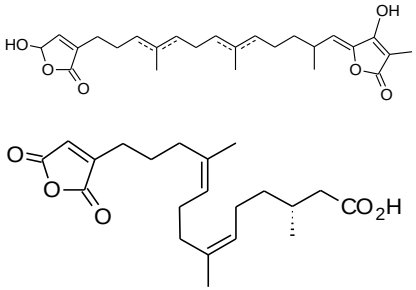
Table 1.1: Summary of synthetic and natural potential inhibitor for MTB ICL and non-MTB ICL.

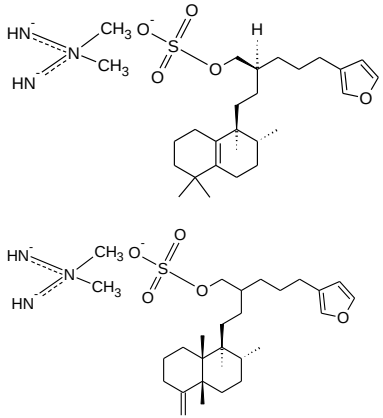
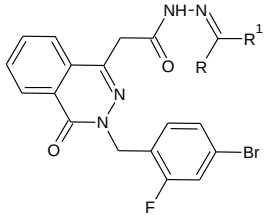
No.	Published Year	Inhibitor	Source	Description	Target ICL	Inhibition* (IC_{50})	Remarks
1	1977	Itaconate (McFadden and Purohit, 1977; Bentrup *et al.*, 1999) 	Synthetic	Succinate analog	*Pseudomonas indigofera*	K_i = 120	Established inhibitor
2	1982	3-nitropropionate (Schloss and Cleland, 1982; Bentrup *et al.*, 1999) 	Synthetic	Succinate analog	*Pseudomonas indigofera*	K_i = 120	Established inhibitor
3	1990	Mycenon (Hautzel *et al.*) 	*Mycena* sp.	Fungi	*Acinetobacter calcoaceticus* *Neurospora crassa* *Ricinus communis*	5.2 μM 7.4 μM	No information on positive control

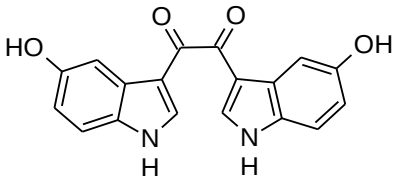
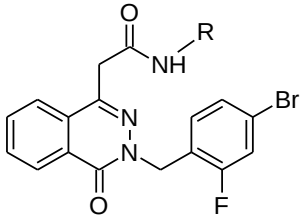
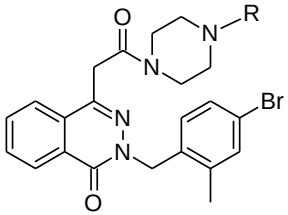
4	1990	3-bromopyruvate (Ko and McFadden, 1990; Bentrup *et al.*, 1999) 	Synthetic	Glyoxylate analog	*Escherichia coli*	3 μM	Established inhibitor
5	2005	DNAzyme (Li *et al.*, 2005)	Synthetic	-	*Mycobacterium tuberculosis*	-	
6	2006	Extract of Traditional Chinese Medicine (Bai *et al.*)	*Zingiber officinale, Illicium verum*	Plant	*Mycobacterium tuberculosis*	47.7 μg/ml 18.2 μg/ml	Positive control IC_{50} of itaconate = 90 μg/ml (Good inhibitory)
7	2007	Hydroquinone derivatives (Yang *et al.*) 	Synthetic	-	*Candida albicans*	0.28-1.02 mM	Positive control IC_{50} of itaconate = 0.06 mM (Weak inhibitory)

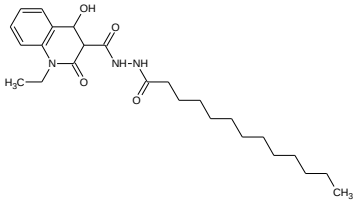
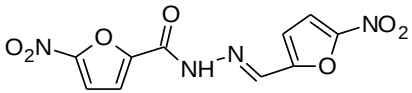
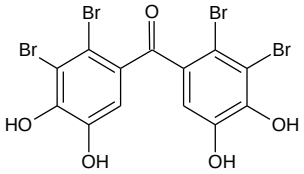
8	2007	Halisulfate 1 (Shin *et al.*, 2007; Lee *et al.*, 2007b) 	*Hippospongia* sp.	Marine sponge	*Magnaporthe grisea*	12.6 μM	No information on positive control (High inhibitory)
9	2007	Bromophenols (Lee *et al.*)  	*Odonthalia corymbifera*	Red algae	*Magnaporthe grisea*	2.0 – 2.8 μM	Positive control IC_{50} of 3-nitropropionate = 94.2 μM (High inhibitory)

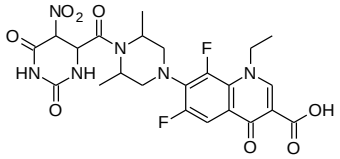
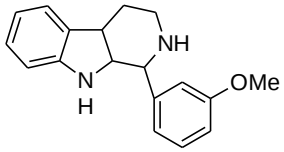
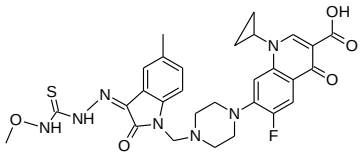
		Polyoxygenated diterpenes (Jang *et al.*)					
10	2008	  	*Phorbas* sp.	Marine sponge	-	LC_{50} of 55 - 140 µg/ml	No information on positive control (Weak inhibitory)
		Meroditerpenoids (Jung *et al.*)					
11	2008	 	*Sargassum siliquastrum*	Brown algae	*Candida albicans*	50 – 95 µg/ml	No information on positive control (Weak inhibitory)

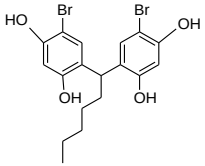
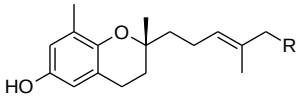
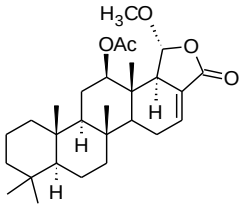
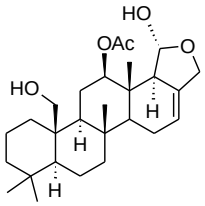
12	2008	Dihydroxystyrene metabolites (Chang *et al.*) 	Association of *Poecillastra wondoensis* and *Jaspis* sp.	Marine sponge	*Candida albicans*	28.7 to >200 µg/ml	Positive control IC_{50} of itaconate = 5.8 µg/ml (Weak to moderate inhibitory)
13	2008	Sesterterpenoids (Wang *et al.*) 	*Sarcotragus* sp.	Marine sponge	*Candida albicans*	12.5 – 19.9 µg/ml	Positive control IC_{50} of Itaconate = 4.9 µg/ml (Moderate to high inhibitory)

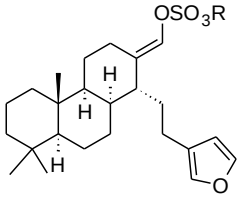
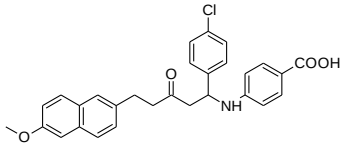
14	2008	Sesterterpene sulphates (Lee *et al.*) 	*Dysidea* sp	Marine sponge	*Candida albicans*	31.3 – 33.8 μM	Positive control IC_{50} of 3-nitropropionate = 50.7 μM (High inhibitory)
15	2009	Pthalazinyl derivatives (Sriram *et al.*, 2009) 	Synthetic	-	*Mycobacterium tuberculosis*	45 – 61% inhibition at 10 μM	Positive control 3-nitropropionate has 63.2% inhibition at 100 μM

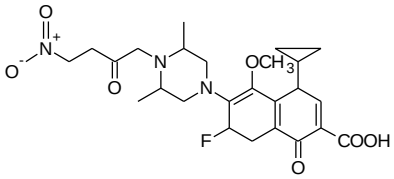
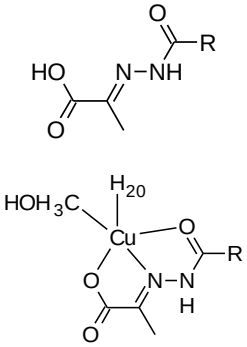
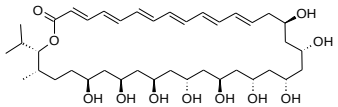
16	2009	Hyrtiosin B (Lee *et al.*) 	*Hyrtios* sp.	Marine sponge	*Candida albicans*	89 μM	Positive control IC_{50} of 3-nitropropionate = 50.7 μM (High inhibitory)
17	2010	Phthalazin-4-ylacetamide (Sriram *et al.*, 2010b)  	Synthetic	-	*Mycobacterium tuberculosis*	40.62 – 66% inhibition at 10 μM	Positive control 3-nitropropionate has 68.2% inhibition at 100 μM

18	2010	Extract of Traditional Chinese Medicine (I2906) (Lu *et al.*, 2010) 	-	Plant	*Mycobacterium tuberculosis*	134 μg/ml	Control samples were untreated samples
19	2010	5-Nitro-2-furoic acid hydrazones with furan-2-carbaldehyde (Sriram *et al.*, 2010c) 	Synthetic	-	*Mycobacterium tuberculosis*	86.8% inhibition at 10 mM	Positive control 3-nitropropionate has 63.2% inhibition at 100 μM (Good inhibitory)
20	2010	Bromophenols (Oh *et al.*) 	Synthetic	-	*Candida albicans*	2.65 μM	Positive control IC_{50} of 3-nitropropionate = 50.7 μM (High inhibitory)

21	2010	5-Nitro-2,6-dioxohexahydro-4-pyrimidinecarboxamides (Sriram *et al.*, 2010a) 	Synthetic	-	*Mycobacterium tuberculosis*	45.7% inhibition at 10 mM	Positive control 3-nitropropionate has 68.2% inhibition at 100 μM (Good inhibitory)
22	2010	Indole-containing natural compound (analog) (Lee *et al.*) 	Synthetic	-	*Candida albicans*	75 μM	Positive control IC_{50} of 3-nitropropionate = 50 μM (High inhibitory)
23	2010	Isatinyl thiosemicarbazones derivatives (Banerjee *et al.*, 2011) 	Synthetic	-	*Mycobacterium tuberculosis*	63.44% inhibition at 10 mM	Positive control 3-nitropropionate has 65.9% inhibition at 100 mM (Good inhibitory)

24	2011	Brominated resorcinol dimer (Bouthenet *et al.*) 	Synthetic	-	*Candida albicans*	28 μM	Positive control IC_{50} of 3-nitropropionate = 6.0 μM (Good inhibitory)
25	2011	Sargachromanols (Chung *et al.*) 	*Sargassum siliquastrum*	Brown algae	*Candida albicans*	118.4 – 172.9 μM	Positive control IC_{50} of 3-nitropropionate = 34.8 μM (Moderate inhibitory)
26	2011	Scalarane Sesterterpenes (Jeon *et al.*)  	*Hyatella* sp.	Marine sponge	*Candida albicans*	40.8 – 55.3 μM	Positive control IC_{50} of 3-nitropropionate = 27.9 μM (Weak inhibitory)

27	2011	Suvanine salt (Bae *et al.*) 	*Coscinoderma* sp.	Marine sponge	*Candida albicans*	5 – 17 μM	Positive control IC_{50} of 3-nitropropionate = 27.9 μM (Moderate inhibitory)
28	2011	Chelerythrine extract (Liang *et al.*)	*Chelidonium majus*	Plant	*Mycobacterium tuberculosis*	Expression level decreased 5 fold	-
29	2011	Mannich base, Ydcm67 (Ji *et al.*) 	Synthetic	-	*Mycobacterium tuberculosis*	57.4% inhibition at 0.05mg/ml	Positive control oxalic acid has 95.55% inhibition at 0.05M
30	2011	Peptide inhibitor (Yin *et al.*)	Synthetic	-	*Mycobacterium tuberculosis*	Inhibition rate 38.2 – 47.92%	Samples contain no peptide inhibitor or ICL in reaction system as controls

31	2011	3-nitropropionamides derivatives (Sriram *et al.*) 	Synthetic	-	*Mycobacterium tuberculosis*	0.1 μM	Positive control IC_{50} of 3-nitropropionate = 116.0 μM (Good inhibitory)
32	2012	Pyruvate-isoniazid analog with their copper complex (Shingnapurkar *et al.*) 	Synthetic	-	*Mycobacterium tuberculosis*	Inhibition rate 6 – 92 %	Control docking using pyruvic acid
33	2012	Bahamaolides A (Macrolide) (Kim *et al.*) 	*Streptomyces* sp.	Actinomycete (Actinobacteria)	*Candida albicans*	10.8 μM	Positive control IC_{50} of 3-nitropropionate = 20.1 μM (High inhibitory)