Novel inhibitors for biotin biosynthesis pathway in *Mycobacterium tuberculosis*

by

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Abbreviations

ACC	Acetyl-CoA carboxylase
ATP	Adenosine 5′-triphosphate
ADP	Adenosine 5′-diphosphate
Apo	Unliganded enzyme
BirA	Biotin inducible repressor A
BLAST	Basic local alignment search tool
bp	Base pair
BPL	Biotin protein ligase
BSA	Bovine serum albumin
CTP	Cytosine 5′-triphosphate
°C	Degrees Celsius
Da	Dalton
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiotheitol
*Ec*DTBS	*Escherichia coli* dethiobiotin synthetase
EDTA	Ethylenediaminetetraacetic acid
FDA	Food and drug administration
GTP	Guanosine 5′-triphosphate
Holo	Ligand bound enzyme
hr	Hour
HTS	High throughput screening
IC_{50}	Inhibition concentration at 50% enzyme activity
ITP	Inosine 5′-triphosphate
kb	kilobase pair
kDa	kilodalton
k_{cat}	Catalytic constant
K_D	Dissociation constant
K_m	Michaelis-Menten constant
K_i	Inhibition constant

LB	Luria broth
MW	Molecular weight
min	Minute, minutes
M	Molar
MIC	Minimal inhibitory concentration
*Mt*DTBS	*Mycobacterium tuberculosis* dethiobiotin synthetase
MW	Molecular weight
MWCO	Molecular weight cut-off
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PDB	Protein data bank
PMSF	Phenylmethylsulfonylfluoride
RMSD	Root mean square deviation
rpm	Revolutions per minute
RU	Resonance units
s	Seconds
SDS	Sodium dodecyl sulphate
SPR	Surface plasmon resonance
TTP	Thymidine 5′-triphosphate
Tris	2-amino-2-hydroxymethylpropane-1,3-diol
UTP	Uridine 5′-triphosphate
V_{max}	Maximum velocity

Abstract

Tuberculosis (TB) ranks alongside HIV-AIDS and malaria as one of the leading causes of death by infectious disease worldwide. The 2015 Millennium Development Goals (MDGs) for reducing the mortality rate and the incidence of new patients to 50% of the 1990 incidences have nearly been reached. However, the prevalence of multidrug-resistant TB (MDR-TB) remains well off-track and needs to be addressed as a public health crisis. Within the past 40 years only one new anti-TB drug with a unique mode of action, namely bedaquiline (FDA approved in 2012), has become available for drug resistant strains of TB but even this has concerning side effects. Clearly, there is an urgent need for safer drugs that have no pre-existing resistance mechanism to terminate the global prevalence of drug resistance TB.

Biotin biosynthesis has been proposed as a promising druggable target for anti-TB drug discovery. Biotin is an essential metabolite required for growth of all living cells. Biotin is synthesized *de novo* in microorganism, plants, and fungi. The absence of this metabolic pathway in humans makes biotin biosynthesis attractive for antibiotic discovery. In particular, biotin biosynthesis plays an important metabolic role as the sole source of biotin in all stages of the tuberculi life cycle due to the lack of biotin transporter. Biotin is intimately associated with lipid synthesis where the products form key components of the cell membrane that is critical for bacterial survival. Therefore, enzymes involving in lipid synthesis and biotin biosynthesis have been designated as an excellent target for the development of new anti-TB drugs to combat drug resistant TB.

Dethiobiotin synthetase (DTBS) catalyzes the penultimate step of the biotin biosynthetic pathway. It was selected as a target for screening inhibitors for *M. tuberculosis* biotin biosynthesis in this study due to its essential role in the growth and virulence of tuberculi. X-ray crystal structures of *M. tuberculosis* DTBS (*Mt*DTBS) reveals two preformed adjacent ligand-binding pockets that allowed DAPA and NTP substrates to bind independently, making both pockets attractive for drug discovery. Enabling technologies were developed for the characterization of DTBS enzymes, including *in silico* screening coupled with X-ray crystal structures and three new facile assays for identifying ligand binding in the NTP pocket, namely an enzyme assay, a competitive ATP-binding assay and surface plasmon resonance (SPR) analysis. Unexpectedly, *Mt*DTBS was shown to have broad specificity for a variety of nucleotide triphosphates, although the enzyme had the highest affinity for CTP in competitive binding and SPR assays. For the first time, X-ray crystal structure of *Mt*DTBS bound to a nucleotide triphosphate (CTP) has been reported, showing that the nucleoside base is stabilized in its pocket through hydrogen-bonding interactions with the protein backbone, rather than amino acid side chains. These novel findings for *Mt*DTBS are in contrast to other DTBS orthologs, for example *Escherichia coli* DTBS (*Ec*DTBS) preferentially binds ATP primarily through hydrogen-bonds between the adenine base and the carboxamide side chain of a key asparagine. Mutational analysis performed alongside *in silico* experiments revealed a gate-keeper role of asparagine at position 175 in *E. coli* DTBS that excludes binding of other nucleotide triphosphates. Due to the absence of the gate-keeper at an equivalent position, *Mt*DTBS thus has the broad specificity to multiple types of nucleotide triphosphates.

The X-ray crystal structure of *Mt*DTBS in complex with CTP was used in an *in silico*, fragment-based approach to drug discovery. Compounds identified by *in silico* docking were verified using an SPR binding assay and DTBS enzyme assay. Total six hits (namely CT6,

CT7, B1, B3, B7, and B9) were identified that were predicted to bind to the protein "hot spot" located in the phosphate-binding loop at the junction of the two ligand binding pockets. Lineweaver-Burk analysis revealed one compound, gemcitabine, was competitive against DAPA and ATP. The low molecular weight (< 300 Da), low chemical complexity, and good ligand efficiency (LE) (0.2-0.3 kcal/mol/heavy atom) of the hits make them attractive targets for chemical development into more drug-like leads. Interestingly, the anti-cancer gemcitabine CT6 clearly showed *in vitro* inhibitory activity against *Mt*DTBS, suggesting an application of this existing drug as a new anti-TB agent. As proof of concept, the potential optimization of leads has been proposed via merging CT6 with DAPA carbamate in order to avoid potential toxicity that might cause through off-target of human NTP-dependent enzymes. Finally, the potential transcriptional regulation of *M. tuberculosis* biotin biosynthesis has been firstly proposed in order to understand the biotin metabolism of tuberculi and to combat TB effectively.

Thesis layout:

The thesis will be presented as a combination of conventional chapters and publication formats. Two manuscripts are presented in chapter 4 and 5 with their own referencing. A general introduction, general materials and method, and final discussion and future direction is also be included to link together all the research conducted during my candidate. A publishing agreement with all co-authors involved with the work is included.

Statement of Originality

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Wanisa Salaemae and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Wanisa Salaemae Date

Communications and Presentations

W. Salaemae, M. Y. Yap, S. W. Polyak, M. C. J. Wilce, G. W. Booker (2013) CTP analogs as novel inhibitors of Dethiobiotin synthetase: Implication for tuberculosis research. *Drug Discovery and Therapy World Congress*, *Boston*, *USA*. Poster Presentation. **Awarded** the Royal Thai Government Scholarship to cover registration, flights and accommodation.

W. Salaemae, M. Y. Yap, S. W. Polyak, M. C. J. Wilce, G. W. Booker (2012) A new approach to combat drug resistant tuberculosis via inhibiting the dethiobiotin synthetase. *Adelaide Protein Group Meeting*, *Adelaide*, *Australia*. Poster Presentation.

W. Salaemae, S. W. Polyak, G. W. Booker (2012) Novel inhibitors of dethiobiotin synthetase from *Mycobacterium tuberculosis* represent an avenue to the discovery of new anti-TB agents. *Australian Society for Medical Research Meeting SA Division Scientific Meeting*, *Adelaide*, *Australia*. Oral Presentation.

W. Salaemae, S. W. Polyak, G. W. Booker (2012) Dethiobiotin synthetase in *Mycobacterium tuberculosis*: A novel drug target. *37th Lorne Proteins Conference*, *Lorne*, *Australia*. Poster Presentation. **Awarded** student registration of $295.

W. Salaemae, S. W. Polyak, G. W. Booker (2011) Novel inhibitors for the biotin biosynthesis pathway in *Mycobacterium tuberculosis*. *Australian Society for Medical Research Meeting SA Division Scientific Meeting*, *Adelaide*, *Australia*. Poster Presentation.

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