Exploring the structure-function relationship of Biotin Protein Ligase from *Staphylococcus aureus*: Implications for selective inhibitor design

by

Tatiana Pereira Soares da Costa

M.Sc. (Honours)

A thesis submitted to the University of Adelaide, South Australia in fulfilment of the requirements for the degree of Doctor of Philosophy

Discipline of Biochemistry
School of Molecular and Biomedical Sciences
University of Adelaide
South Australia

December, 2012
## Table of Contents

Abbreviations........................................................................................................................ iv

Abstract................................................................................................................................ vii

Declaration for thesis containing published work.................................................................x

Publication listing..................................................................................................................xi

Communications and presentations.................................................................................... xiii

Acknowledgements............................................................................................................. xvi

### Chapter 1: Introduction.................................................................................................1

1.1 Need for new antibiotics.............................................................................................2
1.2 Biotin.......................................................................................................................... 3
1.3 Biotin-dependent enzymes and the biotin domain.................................................... 3
1.4 Protein biotinylation is essential in bacteria............................................................. 6
1.5 BPL-catalyzed biotinylation reaction.........................................................................8
1.6 Classes of BPLs..........................................................................................................9
1.7 Characterization of EcBPL........................................................................................11
1.8 SaBPL structure.........................................................................................................15
1.9 Differences between SaBPL and HsBPL................................................................. 18
1.10 BPL inhibitors.........................................................................................................20
1.11 Approaches to drug design..................................................................................... 21
1.12 In situ click chemistry, a novel approach to drug discovery.................................23
1.13 Aims of the project..................................................................................................26
1.14 References...............................................................................................................28

### Chapter 2: Selective inhibition of biotin protein ligase from Staphylococcus aureus.. 35

Statement of authorship..................................................................................................... 36

Published manuscript...................................................................................................... 39
Chapter 3: Biotin analogues with antibacterial activity are potent inhibitors of biotin protein ligase…………………………………………………………………………………58

Statement of authorship…………………………………………………………………………………...59

Published manuscript………………………………………………………………………………………………61

Chapter 4: Optimizing in situ click chemistry: the screening and identification of biotin protein ligase inhibitors……………………………………………………………...82

Statement of authorship………………………………………………………………………………………………83

Manuscript……………………………………………………………………………………………………85

Chapter 5: A novel link between protein homodimerization and inhibitor binding to biotin protein ligase from Staphylococcus aureus………………………………………..105

Statement of authorship…………………………………………………………………………………………….106

Manuscript…………………………………………………………………………………………………………108

Chapter 6: ‘Humanized’ biotin protein ligase provides clues about inhibitor selectivity…………………………………………………………………………………………………….134

Statement of authorship…………………………………………………………………………………………….135

Manuscript…………………………………………………………………………………………………………136

Chapter 7: A novel molecular mechanism to explain biotin-unresponsive holocarboxylase synthetase deficiency……………………………………………………………150

Statement of authorship…………………………………………………………………………………………….151

Published manuscript…………………………………………………………………………………………………153
Chapter 8: Final discussion & future directions .................................................. 166

8.1 Final discussion .............................................................................................. 167

8.1.1 Understanding the differences between BPLs ........................................... 167

8.1.2 Inhibitor design ......................................................................................... 171

8.2 Future directions ......................................................................................... 175

8.2.1 In situ click chemistry ............................................................................... 175

8.2.2 Discovering novel scaffolds to refine the biotin-triazole pharmacophore .. 175

8.2.3 Biotin transporter ...................................................................................... 178

8.2.4 Transcriptional regulation ....................................................................... 180

8.3 Conclusions ................................................................................................. 181

8.4 References ................................................................................................. 182
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AaBPL</td>
<td><em>Aquifex aeolicus</em> biotin protein ligase</td>
</tr>
<tr>
<td>ACC</td>
<td>Acetyl-CoA carboxylase</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>Apo</td>
<td>Unliganded enzyme</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AUC</td>
<td>Analytical ultracentrifugation</td>
</tr>
<tr>
<td>BC</td>
<td>Biotin carboxylase</td>
</tr>
<tr>
<td>BCCP</td>
<td>Biotin carboxyl carrier protein</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BPL</td>
<td>Biotin protein ligase</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>CA-MRSA</td>
<td>Community acquired methicillin resistant <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism</td>
</tr>
<tr>
<td>CT</td>
<td>Carboxyl transferase</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EcBPL</td>
<td><em>Escherichia coli</em> biotin protein ligase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>HA-MRSA</td>
<td>Hospital acquired methicillin resistant <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>HCS</td>
<td>Holocarboxylase synthetase</td>
</tr>
<tr>
<td>Holo</td>
<td>Ligand bound enzyme</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>HsBPL</td>
<td><em>Homo sapiens</em> BPL</td>
</tr>
<tr>
<td>HTS</td>
<td>High-throughput screening</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Inhibition concentration at 50% enzyme activity</td>
</tr>
<tr>
<td>k&lt;sub&gt;cat&lt;/sub&gt;</td>
<td>Catalytic constant</td>
</tr>
<tr>
<td>k&lt;sub&gt;d&lt;/sub&gt;</td>
<td>Off-rate</td>
</tr>
<tr>
<td>K&lt;sub&gt;D&lt;/sub&gt;</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>K&lt;sub&gt;m&lt;/sub&gt;</td>
<td>Michaelis-Menten constant</td>
</tr>
<tr>
<td>K&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Inhibition constant</td>
</tr>
<tr>
<td>M&lt;sub&gt;w&lt;/sub&gt;</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>MCD</td>
<td>Multiple carboxylase deficiency</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimal inhibitory concentration</td>
</tr>
<tr>
<td>MRSA</td>
<td>Methicillin resistant <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>MSSA</td>
<td>Methicillin sensitive <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>MtBPL</td>
<td><em>Mycobacterium tuberculosis</em> biotin protein ligase</td>
</tr>
</tbody>
</table>
NMR  Nuclear magnetic resonance
PAGE  Polyacrylamide gel electrophoresis
PC  Pyruvate carboxylase
PCR  Polymerase chain reaction
PDB  Protein data bank
PhBPL  Pyrococcus horikoshii biotin protein ligase
rpm  Revolutions per minute
RNA  Ribonucleic acid
RU  Resonance units
s  Seconds
SaBPL  Staphylococcus aureus biotin protein ligase
SaPC  Staphylococcus aureus pyruvate carboxylase
ScBPL  Saccharomyces cerevisiae biotin protein ligase
SAR  Structure-activity relationship
SDS  Sodium dodecyl sulphate
SPR  Surface plasmon resonance
Tris  2-amino-2-hydroxymethylpropane-1,3-diol
WT  Wild-type
Abstract

There is a well-documented need to replenish the antibiotic pipeline with new products to combat the rise of drug resistant bacteria, such as the superbug methicillin resistant \textit{Staphylococcus aureus} (MRSA). One strategy to combat drug resistance is to identify new chemical classes with novel mechanisms of action and that are not subject to existing resistance mechanisms. As most of the obvious bacterial drug targets with no equivalents in mammals have been well explored, targets with a closely related human homologue represent a new frontier in antibiotic discovery. However, to avoid potential toxicity to the host, these inhibitors must have extremely high selectivity for the bacterial target over the human equivalent. This thesis is focused upon exploiting the ubiquitous enzyme biotin protein ligase (BPL), which is involved in the essential cellular process of attaching biotin onto biotin-dependent enzymes. Due to the pivotal metabolic roles played by biotin-dependent enzymes in bacteria, BPL has been proposed as a promising new antibiotic target. Hence, BPL inhibitors with selectivity for the bacterial isozyme over the human equivalent promise a new class of antibiotic to combat MRSA.

The aim of this project was to provide proof of concept data demonstrating that BPL from a pathogen could be selectively targeted for inhibition over the human equivalent. Here I employed a combination of structure-guided drug design and fragment-based approaches to discover novel BPL inhibitors. The X-ray crystal structure of \textit{S. aureus} BPL (SaBPL) shows two adjacent binding sites for the ligands biotin and ATP, making it an ideal candidate for a fragment-based approach to drug discovery. Although the residues at the biotin-binding site are highly conserved, the nucleotide pocket shows a high degree of variability that can be exploited to create compounds selective towards BPLs from pathogens. The biotin 1,2,3 triazole analogues identified in this work yielded our most potent and selective inhibitor ($K_i = 90 \text{ nM}$) with $>1100$-fold selectivity for the SaBPL over the human homologue (Chapter 2). The molecular basis for the selectivity was identified using mutagenesis studies with a key arginine residue in the BPL active site necessary for selective binding. Importantly, the biotin triazole inhibitors showed \textit{in vivo} cytotoxicity against \textit{S. aureus}, but not cultured mammalian cells (Chapter 2).

In an attempt to identify new chemical scaffolds with improved ligand efficiency for chemical development, a series of analogues based on the natural ligand biotin were also
designed and tested for enzyme inhibition and antimicrobial activities against clinically relevant strains of *S. aureus* (Chapter 3). This approach resulted in highly potent compounds ($K_i < 100$ nM) with antibacterial activity against MRSA strains (MIC = 2 - 16 µg/mL). Whilst only moderate selectivity over the human enzyme (10 - 20 fold) was observed, the biotin analogues provided a suitable chemical scaffold with high ligand efficiency for further chemical development. One of the compounds identified was biotin acetylene, which forms a long lived complex with *SaBPL* and is a precursor for *in situ* click reactions. This target-guided approach to drug discovery relies on the ability of the enzyme to choose its own inhibitors from a range of acetylene and azide building blocks to form specific triazole products. Since a class of biotin-triazole molecules had already been identified as selective inhibitors of *SaBPL*, we reasoned that this enzyme would provide an ideal candidate for performing *in situ* click approach to inhibitor discovery. In this work, a protocol for the BPL-catalyzed *in situ* click reaction was optimized to select the optimum triazole-based inhibitor using biotin acetylene as an anchor molecule to recruit complimentary fragments that could bind in the peripheral ATP pocket (Chapter 4). The *in situ* reaction was shown to be improved by the use of a *SaBPL* mutant that promoted diffusion of the triazole product from the active site following synthesis. This novel approach improved efficiency and ease of detection (Chapter 4).

Apart from drug discovery, this thesis also focuses on enzymatic characterization of *SaBPL* and highlighting the key differences between *SaBPL* and the human homologue. The structure of human BPL is yet to be reported, so structure-function studies were performed to elucidate new information about the bacterial and human enzymes. The oligomeric state of *SaBPL* was investigated using analytical ultracentrifugation in its apo form and in the presence of ligands (Chapter 5). Unlike human BPL, *SaBPL* was shown to dimerize in solution. A single amino acid in *SaBPL*, Phe123, was identified to have a dual key role in dimer formation and inhibitor binding (Chapter 5).

One of the major roadblocks to obtaining crystals of the full-length human enzyme is the low yield of protein obtained from recombinant expression and purification. In this thesis, an alternative approach is described that could be used to increase our chances of obtaining structural data about the human BPL. I created a ‘humanized’ chimeric protein in which all seven residues in the nucleotide pocket of *SaBPL* that are not conserved with the human BPL were mutated to their human equivalents. This ‘humanized’ protein exhibited similar
kinetic and inhibition properties to the human enzyme (Chapter 6). Crystal trials have commenced to help direct future drug development efforts. Further studies on the human BPL enzyme will also be described, including the dissection of the binding mechanism using surface plasmon resonance (Chapter 7). The N-terminal domain of this enzyme was shown to play a role in stabilizing the complex between the enzyme and the biotin domain substrate, providing the first molecular explanation for human BPL-deficient patients that do not respond to biotin therapy.

In summary, this work demonstrates for the first time that BPL from the clinically important pathogen *Staphylococcus aureus* can be selectively inhibited. A provisional patent has been filed for the biotin 1,2,3 triazole molecules I have identified. These discoveries will enable further development of a new class of antibiotics.

Thesis layout:

The thesis will be presented as a series of manuscripts either published, submitted or to be submitted for publication. Each manuscript will be a chapter with its own references. A general introduction and discussion will also be included to link together all the research conducted during candidature. A publishing agreement with all co-authors involved with the work is also included.
Declaration for thesis containing published work and/or work prepared for publication

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 Tatiana Pereira Soares da Costa 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.

I give consent to this copy of my thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

The author acknowledges that copyright of published works contained within this thesis (as listed on the next page) resides with the copyright holder(s) of those works.

I also give permission for the digital version of my thesis to be made available on the web, via the University’s digital research repository, the Library catalogue and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

................................................................. ........................................
Tatiana Pereira Soares da Costa Date
I acknowledge the copyright of published works contained within this thesis including:

Chapter 2:


Chapter 3:


Chapter 4:


Chapter 5:

Chapter 6:


Chapter 7:


Authorization to publish each paper has been given and provided in print for each chapter containing copyright and co-authored work, including acknowledgement of contribution to the work from each author.
Communications and Presentations


Acknowledgements

Firstly, I would like to express my sincere gratitude to my supervisors Assoc. Prof. Grant Booker, Dr. Steven Polyak and Prof. Andrew Abell for the continuous support, patience, motivation, enthusiasm and immense knowledge.

Grant, thank you for giving me the opportunity to pursue my PhD studies in your lab and for helping me grow as a scientist. Steven, I’m so grateful that you invited me to visit the lab when I was still in New Zealand. Your extensive knowledge about BPL and all biotin-related things never cease to amaze me! You were always there for me with a big smile on your face and you were always happy to bounce ideas off. Thanks for introducing me to sparkling red wine and for providing so much entertainment every year at the School dinner☺. Andrew, thanks for helping me with all chemistry-related things and for always being so encouraging.

To Prof. John Wallace, you are an inspiration. Thanks for sharing your wisdom with me. You have always been so supportive. Thank you for all your helpful comments and for reading manuscripts.

A big thanks also goes to the present and past members of the lab. Lungisa, thanks for always being there for me, I’m so glad I got to share this experience with you! Big kudos to Ashleigh (the most photographic person I know☺), Martin (who owes me a year’s supply of Nutella☺), Mike (a.k.a. the mini-prep king), Tony (who introduced me to Korean food), Angie (the award-winning baking queen), Kate (the cool mum☺), Karen, Al, Sushil, Connie and Ethan. Thanks also to the members of the BPL team in the Department of Chemistry for synthesizing compounds for me in record time, namely Dr. William Tieu, Dr. Ondrej Zvarec and Kelly Keeling.

Thanks to our collaborators Prof. Mathew Wilce and Min Yap at Monash University for providing us with beautiful crystal structures. I also would like to thank Assoc. Prof. Matthew Perugini for allowing me to come to his lab and learn about the wonderful world of analytical ultracentrifugation. Thanks also for the Perugini crew for making me feel so welcome during my stay in Melbourne.
Thanks to all the friendly people at the School of Molecular and Biomedical Science, including teaching staff, everyone in the CSU and the store. Particular thanks to Dr. Chris Wong, Dr. James Botten, Dr. Tony Fratini and Lynn Rogers for sharing their love of teaching with me and for giving me the opportunity to be involved in undergraduate teaching during my studies.

I’m very appreciative for all the support from my friends and family and for keeping me sane during my studies. Thanks to Gabby, Bruno, Shaun and Dan for the constant encouragement and for cheering me up just when I needed it. A special thanks to Shaun for being my rock during thesis writing.

Lastly, I would like to dedicate this thesis to my Mum and Dad for the never-ending support throughout my years at university and for always believing in me.