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

by

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Abbreviations

*Aa*BPL	*Aquifex aeolicus* biotin protein ligase
ACC	Acetyl-CoA carboxylase
AMP	Adenosine monophosphate
Apo	Unliganded enzyme
ATP	Adenosine triphosphate
AUC	Analytical ultracentrifugation
BC	Biotin carboxylase
BCCP	Biotin carboxyl carrier protein
bp	Base pair
BPL	Biotin protein ligase
BSA	Bovine serum albumin
^{o}C	Degrees Celsius
CA-MRSA	Community acquired methicillin resistant *Staphylococcus aureus*
CD	Circular dichroism
CT	Carboxyl transferase
Da	Dalton
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiotheitol
*Ec*BPL	*Escherichia coli* biotin protein ligase

EDTA	Ethylenediaminetetraacetic acid
FDA	Food and Drug Administration
HA-MRSA	Hospital acquired methicillin resistant *Staphylococcus aureus*
HCS	Holocarboxylase synthetase
Holo	Ligand bound enzyme
HPLC	High-performance liquid chromatography
*Hs*BPL	*Homo sapiens* BPL
HTS	High-throughput screening
IC_{50}	Inhibition concentration at 50% enzyme activity
k_{cat}	Catalytic constant
k_d	Off-rate
K_D	Dissociation constant
K_m	Michaelis-Menten constant
K_i	Inhibition constant
M_w	Molecular weight
min	Minute
M	Molar
MCD	Multiple carboxylase deficiency
MIC	Minimal inhibitory concentration
MRSA	Methicillin resistant *Staphylococcus aureus*
MSSA	Methicillin sensitive *Staphylococcus aureus*
*Mt*BPL	*Mycobacterium tuberculosis* biotin protein ligase

NMR	Nuclear magnetic resonance
PAGE	Polyacrylamide gel electrophoresis
PC	Pyruvate carboxylase
PCR	Polymerase chain reaction
PDB	Protein data bank
*Ph*BPL	*Pyrococcus horikoshii* biotin protein ligase
rpm	Revolutions per minute
RNA	Ribonucleic acid
RU	Resonance units
s	Seconds
*Sa*BPL	*Staphylococcus aureus* biotin protein ligase
*Sa*PC	*Staphylococcus aureus* pyruvate carboxylase
*Sc*BPL	*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 *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 *S. aureus* BPL (*Sa*BPL) 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 nM) with >1100-fold selectivity for the *Sa*BPL 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 *in vivo* cytotoxicity against *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 *Sa*BPL 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 *Sa*BPL, 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 *Sa*BPL 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 *Sa*BPL and highlighting the key differences between *Sa*BPL 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 *Sa*BPL was investigated using analytical ultracentrifugation in its apo form and in the presence of ligands (Chapter 5). Unlike human BPL, *Sa*BPL was shown to dimerize in solution. A single amino acid in *Sa*BPL, 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 *Sa*BPL 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.

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T. P. Soares da Costa, M. Y. Yap, W. Tieu, M. C. Wilce, S. W. Polyak , A. R. Abell, G. W. Booker (2011) Exploring the structure-function relationship of biotin protein ligase from *Staphylococcus aureus* to help the development of novel, highly potent and selective inhibitors. *ComBio Conference, Cairns, Australia.* Oral Presentation. **Awarded** student registration bursary of $330 by ASBMB.

T. P. Soares da Costa, M. Y. Yap, W. Tieu, M. C. Wilce, J. C. Wallace, A. R. Abell, S. W. Polyak, G. W. Booker (2011) First evidence of highly potent and selective inhibitors of MRSA biotin protein ligase. *Australian Society for Medical Research Meeting SA Division Scientific Meeting, Adelaide, Australia*. Oral Presentation.

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