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Enzyme targets for drug design of new anti-virulence therapeutics

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Society has benefitted greatly from the use of antibiotics. Unfortunately, the misuse of these valuable molecules has resulted in increased levels of antibiotic resistance, a major global and public health issue. This resistance and the reliance on a small number of biological targets for the development of antibiotics emphasizes the need for new targets. A critical aspect guiding the development of new antimicrobials through a rational structure-guided approach is to understand the molecular structures of specific biological targets of interest. Here we give an overview of the structures of bacterial virulence enzyme targets involved in protein folding, peptidoglycan biosynthesis and cell wall modification. These include enzymes of the thiol-disulphide oxidoreductase pathway (DSB enzymes), peptidyl-proly *cis/trans* isomerases (Mips), enzymes from the Mur pathway and enzymes involved in lipopolysaccharide modification (EptA and ArnT). We also present progress towards inhibitor design of these targets for the development of novel anti-virulence therapeutic agents.

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Introduction

The discovery, development and utility of antibiotics to treat bacterial infections has arguably been one of the most successful public health activities of the 20th century resulting in a dramatic increase in the quality of life for mankind. Antibiotics have been developed to treat a wide variety of Gram-positive and Gram-negative bacterial infections but their successful use has come at a cost, with an increasing number of reports of bacteria found to be resistant to current antibiotic therapies. Concurrently with this increase in antibiotic resistance there has been a decrease in the rate of development of new antimicrobials from the pharmaceutical industry [1,2], resulting in a significant antibiotic crisis. Resistance has been reported for all current antibiotics including the β-lactams, glycopeptides, aminoglycosides, macrolides, quinolones, tetracyclines and alarmingly, even the last line antibiotics such as polymyxin [3]. As a result, the World Health Organisation and the Centres for Disease Control and Prevention in the USA have released a series of reports highlighting the global priority list of antimicrobial resistant pathogens and the urgency for the development of new treatment strategies including drug discovery to circumvent the problem with increasing antibiotic resistance [4–6,7•]. One area of focus is to target bacterial virulence factors which are the cause of pathogenicity in the organism. Anti-virulence factors act to disarm the pathogen enabling clearance by the host immune system. Structures of new targets provide an important tool to assist in the development of novel antibacterial agents, allowing for screening and optimization using both fragment-based methods as well as established small molecule libraries. Two areas where development of new types of antimicrobials have been enhanced through the use of structural studies include enzymes involved in protein folding pathways as well as peptidoglycan biosynthesis and cell wall modification. Here we review the structures of a number of enzyme targets in these different pathways. Disulphide interchange and macrophage infectivity potentiator enzymes have already progressed with inhibitor design studies while the enzymes involved in peptidoglycan biosynthesis such as those of the Mur pathway and endotoxin modifying enzymes such as ArnT and EptA are more novel targets which are certain to be the extensive subject of further studies into inhibitor design.

Disulphide interchange enzymes

Protein synthesis and correct folding often require the assistance of various chaperones including foldases (Hsp70 and Hsp100) [8], oxidoreductases and peptidyl proline *cis/trans* isomerases. Thiol-disulphide oxidoreductases (TDORs) are a large superfamily of enzymes that catalyze the oxidation, reduction, and isomerization of protein disulphide bonds [9]. All members of this family have an active site motif consisting of two Cys residues

separated by two amino acids (CXXC) embedded in a thioredoxin-like fold [10]. TDORs are multi-functional and are widely dispersed in Gram-negative bacteria but have only recently been identified in Gram-positive bacteria through their involvement with specialized processes such as sporulation [11]. While cytoplasmic TDORs, such as thioredoxin, maintain a reducing environment inside the cell, extracytoplasmic TDORs in the cell envelope participate in a variety of physiological processes and in many bacterial pathogens they are essential for virulence [12].

The development of anti-virulence strategies by inhibiting TDORs has focused especially on the oxidative and isomerization periplasmic protein folding pathway in Gram-negative bacteria. The oxidation pathway consists of the disulphide bond (Dsb) proteins DsbA and DsbB, responsible for the catalysis of disulphide bond formation between adjacent Cys residues of protein substrates while the isomerization pathway (DsbC/DsbD system) re-shuffles any incorrectly formed disulphide bonds (Figure 1a). DsbA is the primary disulphide donor that interacts with these substrates and is kept in an oxidized and active state by the membrane bound DsbB [13]. DsbA is a monomeric 21 kDa periplasmic enzyme consisting of the thioredoxin fold containing an extra alpha-domain (65 residues forming four α-helices) inserted into the center of the thioredoxin domain (Figure 1b). The α-domain forms a globular-like cap over the active site CXXC motif located at the N-terminus of the first α-helix of the thioredoxin domain [14]. The redox activity of the enzymes is highly modulated by the presence of a *cis* proline containing loop that makes van der Waals interactions with the active site cysteine residues [15]. A hydrophobic groove, critical for binding to DsbB during the catalytic cycle, has been targeted as a potential inhibitor binding site [16•,17]. DsbB is a cytoplasmic membrane protein and a member of the vitamin K 2,3-epoxide reductase (VKOR) superfamily; it contains four trans-membrane α-helices connected by two periplasmic loops (P1 and P2) that house two cysteine pairs (Cys41–Cys44 and Cys104–Cys130 respectively) essential to DsbA oxidation [18,19] (Figure 1c). The NMR structure of DsbB [20] as well as the crystal structure of the complex of DsbA and DsbB gives important insights into the interaction of the two proteins and the mechanism of rapid DsbB-mediated oxidation of DsbA [21•,22••]. The transfer of electrons from DsbA to DsbB is initiated by interaction of the P2 loop of DsbB with the open hydrophobic groove of reduced DsbA (Figure 1c). This induces a conformational change in the P2 loop of DsbB so that Cys104–Cys130 are separated spatially, and brought into contact with the reduced cysteines (Cys30 and Cys44) of the DsbA active site, enabling a rearrangement of the disulphide bonds and the subsequence electron transfer to the ubiquinone moiety bound to DsbB through the formation of a Cys44-ubiquinone charge transfer complex.

DsbC is a V-shaped homodimer consisting of two 23 kDa monomers (Figure 1d). The monomers consist of a C-terminal thioredoxin domain and an N-terminal dimerization domain connected by a linker sequence [23•]. The active site residues in the thioredoxin domain are positioned so as to face the inside hydrophobic surface of the V-shaped homodimeric structure and prevents interaction with DsbB [23•]. Oxidized DsbC is activated by reduction from the inner membrane bound DsbD which mediates electron transfer from cytoplasmic thioredoxin [24]. DsbD consists of a periplasmic N-terminal domain with an immunoglobulin-like fold (DsbDα), a hydrophobic core that contains eight transmembrane helices (DsbDβ) and periplasmic C-terminal domain (DsbDγ) with a thioredoxin-like fold. The sequence of electron transfer through these domains is DsbDβ → DsbDγ → DsbDα [25] (Figure 1a). The electrons are then further transferred from DsbDα to DsbC. The structure of the DsbC–DsbDα domain shows two asymmetric binding sites with the primary binding site forming an intermolecular disulphide bond between Cys98 of DsbC and Cys109 of DsbDα [26••] (Figure 1d). Furthermore, the DsbC binding cleft undergoes conformational changes upon DsbDα binding to a closed conformation which has led to a model for the interaction of DsbC to other protein substrates for isomerization activity.

Although the Dsb pathway has not been found to be essential in Gram-negative bacteria [9,27], inactivation of the pathway has been shown to attenuate all Gram-negative pathogens investigated to date [2,28]. The structures of the different components of the Dsb pathway open up possibilities for structure-guided design of anti-virulence agents that will assist the innate immune system in clearing the bacterial infection. Anti-virulence compound design has focused mainly on the oxidation pathway (DsbA, DsbB). Rational target-based drug design (RTBDD) strategies [28] have successfully identified novel small molecules that inhibit the function of DsbA and DsbB. Using a combination of STD-NMR-based fragment screening followed up by chemical elaboration, small molecules have been identified [29••] that bind to the active site hydrophobic groove, inhibiting DsbA activity in *Escherichia coli* by blocking the DsbB peptide binding site [16•] (Figure 1e). These studies provide a starting point for further inhibitor development as anti-virulence agents. Because of the structural groups of DsbA [30], opportunities may exist to create specialized inhibitors that target *Enterobacteriaceae*, an important group of pathogens most related to the spread of antimicrobial resistance.

Peptidyl-proly *cis/trans* isomerase enzymes

A novel group of enzymes involved in protein folding and essential for bacterial survival are the macrophage infectivity potentiator (Mip) proteins. Part of the immunophilin superfamily, Mip is a FK506 binding protein (FKBP)