Thanatin – an Insect-derived Antimicrobial Peptide Active against Pathogenic Gram-negative Bacteria

Susan J. Tzotzos

Apeptico Research & Development GmbH, 136 Mariahilfer Strasse, 1150 Vienna, Austria; e-mail: s.tzotzos@apeptico.com

Gram-negative bacteria are particularly efficient in their ability to acquire resistance to antibiotics due to the structure and composition of their cell envelope, which is characterized by the presence of an outer membrane (OM) surrounding the cytoplasmic (inner) membrane (IM) and the peptidoglycan cell wall. The OM is a unique asymmetric bilayer with an inner leaflet consisting of phospholipid and an outer leaflet consisting of lipopolysaccharide (LPS). The OM and LPS of Gram-negative bacteria render them impervious to antibiotics and other toxic compounds such as detergents. It is estimated that Gram-negative bacteria are the cause of more than 30% of hospital-acquired infections, 47% of ventilator-associated pneumonia cases and 45% of urinary tract infections. Thus, there is a continuous pressing need to discover new antibiotics effective against Gram-negative bacteria.

The host defence cationic antimicrobial peptide (AMP), thanatin (from the Greek, "thanatos" which means "death"), first isolated some 20 years ago from the hemipteran insect, the spined soldier bug, *Podisus maculiventris,* displays potent bactericidal activity, especially against extended-spectrum-beta-lactamase (ESBL) producing *Escherichia coli* (Ma B *et al*, 2016). Apart from *E. coli*, antimicrobial activity for thanatin has been reported against the Gram-negative bacteria *Salmonella typhimurium*, *Klebsiella pneumoniae* and *Enterobacter cloacae* with weaker activity against *Erwinia carotovora* and *Pseudomonas aeruginosa*. Thus thanatin is a promising candidate to treat nosocomial infections caused by antibiotic resistant forms of these pathogenic bacteria. Thanatin has also shown antimicrobial activity against some Gram-positive bacteria and other microorganisms (Sinha *et al*, 2017).

Thanatin is composed of 21 amino acid residues (GSKKPVPIIYCNRRTGKCQRM), with an intramolecular disulfide bridge between the two cysteines, Cys11 and Cys18, and has been shown to have high stability in blood plasma (Ma B *et al*, 2016). Free thanatin assumes a beta-hairpin structure for the central sequence (residues I8-M21), with an N-terminal extended region (Fig. 1); in contrast, thanatin in complex with LPS micelles has been demonstrated to adopt a higher order structural assembly of a dimeric four-stranded beta sheet (Sinha *et al*, 2017).
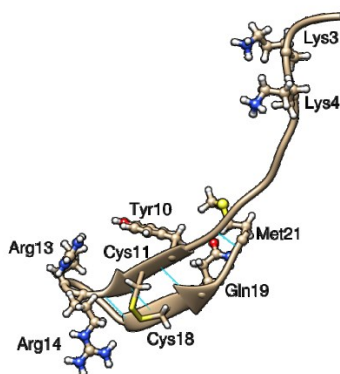
Fig. 1. Representative structure of thanatin in free solution, at 25 °C, pH 5 (PDB ID:5xo4). Thanatin assumes a monomeric beta-hairpin structure with an extended N-terminus. H-bonds shown as light blue lines (Sinha *et al*, 2017).

In a recent report, Swiss researchers have revealed the mechanism of action of thanatin by which it targets the intermembrane protein complex required for LPS transport in *E. coli*. Employing a series of elegant techniques directed at increasing levels of magnification and ultimately, structural determination by NMR, Vetterli and co-workers show how thanatin interferes with the assembly of the seven-member macromolecular protein complex responsible for LPS transport from the IM to the OM (Vetterli *et al*, 2018).

The work of Vetterli and coworkers, builds on earlier work of Freinkman *et al* (2012), who showed by photocrosslinking that LPS transport proteins, LptA-G, form a macromolecular complex, which functions as a protein bridge between the IM and the OM. Freinkman and co-workers defined the architecture of the transenvelope bridge by means of which LPS molecules assembled at the IM are transported in an ATP-dependent manner across the periplasm to the OM. One protein in particular, LptA, forms head to tail oligomers (Suits *et al*, 2008) which span the periplasm (Fig. 2).
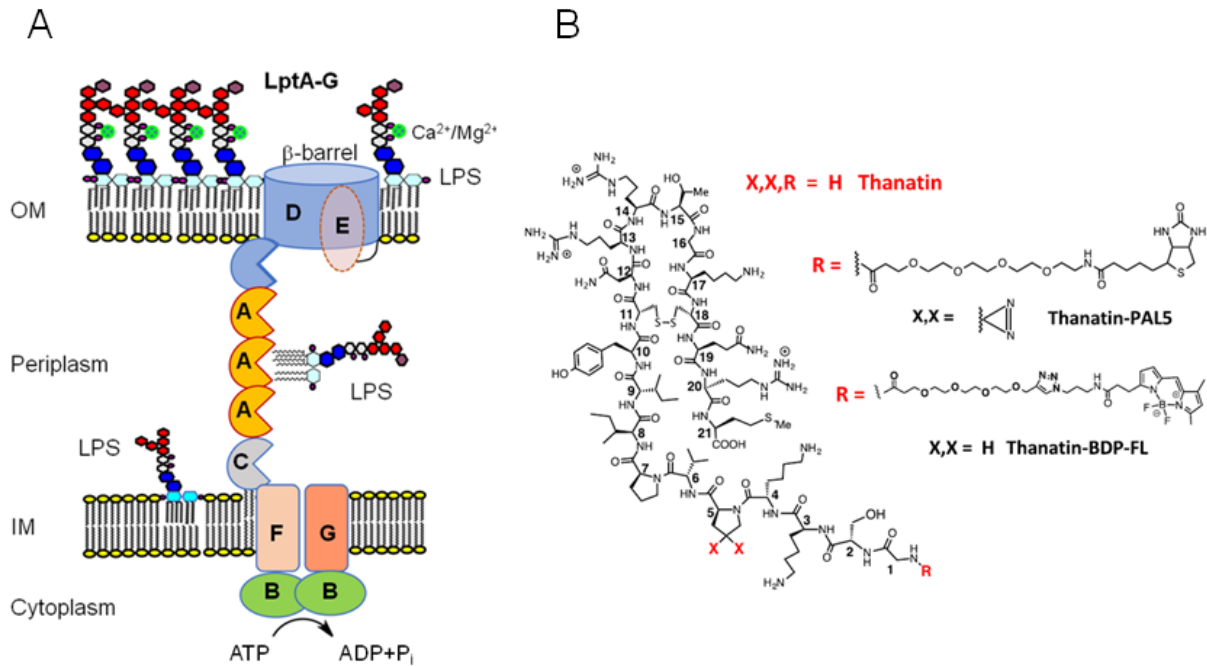
Fig. 2. LPS transport pathway and thanatin-based probes. The LPS transport apparatus in Gram-negative bacteria comprises the seven proteins LptA to LptG, which form a macromolecular complex spanning the IM and OM. LPS transport across the periplasm occurs over a bridge formed by one or more copies of LptA. ADP, adenosine 5′-diphosphate. (**B**) Structures of thanatin and the photoprobe thanatin-PAL5 and fluorescence probe thanatin-BDP-FL (From Vetterli *et al*, 2018).

The work of Vetterli *et al* (2018) can be described at five levels of increasing resolution of cell components:
1) Genetic analysis of *E. coli* thanatin-resistant mutants
2) TEM studies of *E.coli* grown in concentrations of thanatin inhibitory to growth
3) Photoaffinity labelling experiments
4) *In vitro* binding studies using fluorescently-labelled thanatin and LptA
5) NMR structure determination of the thanatin-LptA complex

GENETIC ANALYSIS OF E. COLI THANATIN-RESISTANT MUTANTS

By applying selection for spontaneous thanatin resistant mutants (Than[R]) of *E. coli*, several stably resistant mutants were obtained and subjected to whole genome sequencing. Than[R] mutants showed variation at several positions compared to wild-type (WT), but mutation in the LptA gene was a common feature in all mutants. The genome sequence of one mutant differed from the WT at one position only and that was in the LptA gene. This genetic analysis indicated that thanatin targets the LptA protein of *E.coli.*

TEM STUDIES OF E.COLI GROWN IN CONCENTRATIONS OF THANATIN INHIBITORY TO GROWTH

TEM studies of *E.coli* grown in concentrations of thanatin inhibitory to growth revealed that the antibiotic causes deformation of the cell membrane architecture with accumulation of folds of membrane-like material in the cell interior. Similar folds have been seen when LPS transport is inhibited in *E. coli* by down-regulation of LptA/B, LptC, or LptD.

PHOTOAFFINITY LABELLING EXPERIMENTS

The interaction of thanatin with OM proteins (OMPs) in *E. coli* was tested directly by photoaffinity labelling experiments. Thanatin-derived photoprobes retaining the bactericidal activity of thanatin (thanatin-PAL5 and thanatin-PAL7) were synthesized containing photoproline (in place of Pro5 or Pro7), together with an N-terminal polyethylene glycol linker and biotin tag for pull-down assays. Western Blot analysis of the photolabelled membrane protein extract showed a molecular mass shift of one band between reducing and non-reducing conditions, characteristic of that reported for *E. coli* LptD in the disulfide-reduced and disulfide-oxidized forms.

Thanatin interaction partners were also identified by using the photolabelling strategy in combination with MS-based proteomic analysis which enabled quantification of the relative abundance of proteins in thanatin-PAL5 versus unlabelled control sample. Relative quantitative comparison revealed the specific and photolabelling-dependent enrichment of three OMPs, namely, LptD, LptA, and BamB, of which LptD and LptA were the most significant.

## IN VITRO BINDING STUDIES USING FLUORESCENTLY-LABELLED THANATIN AND LptA

A recombinant full-length LptA [with a His6 tag fused to the C-terminus (LptA-His6)] was produced in *E. coli* and purified to apparent homogeneity by SDS-PAGE. The binding of a fluorescent thanatin probe, thanatin-BDP-FL (Figure 2B) to LptA-His6 was then studied by fluorescence polarization (FP) and of thanatin binding to LptA labeled with DyLight650 by thermophoresis. Control experiments using the enantiomeric form of thanatin (comprising all D-amino acids) resulted in no interaction of D-thanatin with LptA as observed by FP. These binding studies showed that thanatin binds *in vitro* to both LptA and LptD/E in the low nanomolar range.

NMR STRUCTURE DETERMINATION OF THANATIN-LptA COMPLEX

Complex formation between thanatin and a non-aggregating form of LptA (Lpt$_m$) was monitored by [^{15}N,^{1}H]-heteronuclear single-quantum coherence (HSQC) nuclear magnetic resonance (NMR) spectroscopy. The structure of the thanatin-LptA$_m$ complex was determined by mulitdimensional NMR methods in nuclear Overhauser effect spectroscopy (NOESY) experiments. The structure shows that the N-terminal strand of the thanatin beta-hairpin (Pro7-Asn12) docks in parallel orientation onto the first (N-terminal) beta-strand of the beta-jellyroll of LptA$_m$ (Pro35-Ser40). After the beta-turn in thanatin, the C-terminal residues are mostly solvent-exposed (Figure 3), with the exception of the hydrophobic side-chain of Met21, which fits into a hydrophobic pocket on the surface of LptA$_m$. Side chains of Asn12 and Arg13 are likely involved in hydrogen bonding and electrostatic interactions, respectively, with LptA$_m$, and the amide side-chain protons of Asn12 are suitably disposed to form hydrogen bonds with the backbone carbonyl groups of Ser40 and Asp41 (Fig. 3A). Salt bridge formation is possible between the guanidium groups of Arg13 and Arg14 in thanatin and the carboxylate groups of Glu39 and Asp41, respectively, of LptA$_m$ (Fig. 3A).

The NMR structure of LptA complexed with thanatin when superimposed on the structure of the head-to-tail oligomer of LptA, shows that thanatin binding overlaps the site of interaction between LptA subunits and therefore would block this interaction *in vivo* (Fig. 3B).

The C-terminal strands of LptA bind to the N-terminal beta-strands in the periplasmic beta-jellyroll domain of LptD. Intriguingly, the proteins LptA and LptD have a high degree of sequence similarity in their N-terminal beta strands, suggesting that thanatin should also interact with the N-terminal beta-strands in the beta-jellyroll of LptD/E, inhibiting interaction of LptA with LptD.
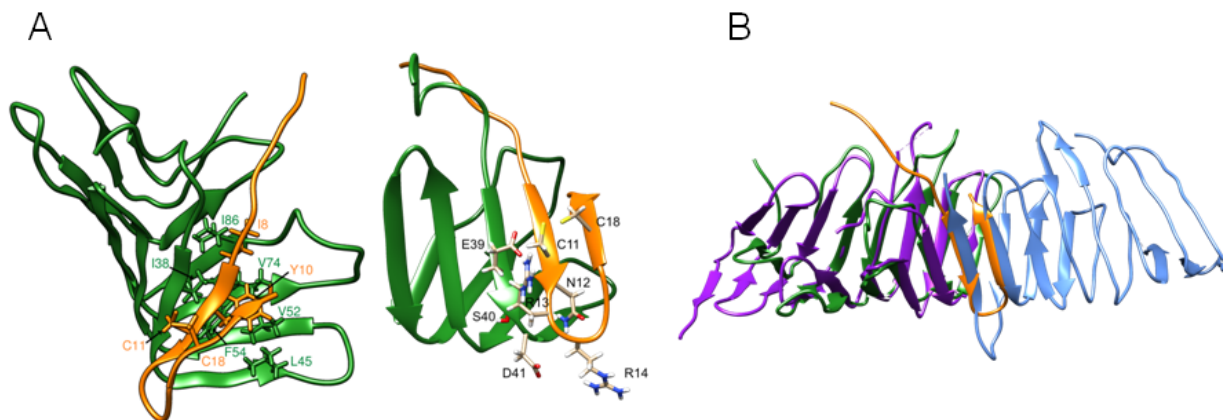
Fig. 3. (**A**) Ribbon model of the LptA$_m$-thanatin complex (PDB ID:6GD5). Residues involved in the protein-peptide hydrophobic interface (left) and in hydrogen bonding and electrostatic interactions (right) are indicated by stick representation. (**B**) Superposition of the LptA dimer (PDB ID: 2R1A); chains B (light blue) and C (purple) with the LptA$_m$-thanatin complex (green/orange). Thanatin occupies a binding site on LptA$_m$ which is used to mediate LptA-LptA interactions needed to form the periplasmic bridge connecting IM and OM for LPS transport (Adapted from Vetterli *et al*, 2018).

CONCLUSION

Vetterli and co-workers revealed a link between the antimicrobial activity of thanatin and LptA and LptD and point to LptA and LptD as interaction partners – biological targets for thanatin in *E. coli*. A new mechanism for antibiotic action is presented: targeting the dynamic network of protein-protein interactions required for assembly of the Lpt complex in *E. coli*.

Thanatin, a naturally occurring peptide, has been identified as a starting point for the development of potential drug candidates targeting deadly and highly problematical bacterial pathogens which cause nosocomial infections.

Additionally, we are given a tantalising insight into the biological function of thanatin in its natural context as an insect defence peptide: controlling numbers of symbiotic or invading bacterial pathogens in the mid-gut of various insect species.

REFERENCES

Freinkman E, Okuda S, Ruiz N, Kahne D. Regulated assembly of the transenvelope protein complex required for lipopolysaccharide export. Biochemistry. 2012 Jun 19;51(24):4800-6. doi: 10.1021/bi300592c. Epub 2012 Jun 8. PubMed PMID: 22668317.

Ma B, Niu C, Zhou Y, Xue X, Meng J, Luo X, Hou Z. The Disulfide Bond of the Peptide Thanatin Is Dispensible for Its Antimicrobial Activity In Vivo and In Vitro. Antimicrob Agents Chemother. 2016 Jun 20;60(7):4283-9. doi: 10.1128/AAC.00041-16. Print 2016 Jul. PubMed PMID: 27161645.

Sinha S, Zheng L, Mu Y, Ng WJ, Bhattacharjya S. Structure and Interactions of AÂ Host Defense Antimicrobial Peptide Thanatin in Lipopolysaccharide Micelles Reveal Mechanism of Bacterial Cell Agglutination. Sci Rep. 2017 Dec 19;7(1):17795. doi: 10.1038/s41598-017-18102-6. PubMed PMID: 29259246.

Suits MD, Sperandeo P, Dehò G, Polissi A, Jia Z. Novel structure of theconserved gram-negative lipopolysaccharide transport protein A and mutagenesis analysis. J Mol Biol. 2008 Jul 11;380(3):476 88. doi: 10.1016/j.jmb.2008.04.045. Epub 2008 Apr 26. PubMed PMID: 18534617.

Vetterli SU, Zerbe K, Müller M, Urfer M, Mondal M, Wang SY, Moehle K, Zerbe O, Vitale A, Pessi G, Eberl L, Wollscheid B, Robinson JA. Thanatin targets the intermembrane protein complex required for lipopolysaccharide transport in Escherichia coli. Sci Adv. 2018 Nov 14;4(11):eaau2634. doi: 10.1126/sciadv.aau2634. eCollection 2018 Nov. PubMed PMID: 30443594.