New anti-infective compounds against *Staphylococcus aureus*

(Edited version of the dissertation)

By
Aparna Viswanathan Ammanath
aus Thrissur Kerala/ Indien

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Preparatory statement

This publication is an edited version of my submitted dissertation “New anti-infective compounds against Staphylococcus aureus” that was accepted by the faculty on 14.12.2022 and published on TOBIAS-lib on 17.01.2023. Sections from this thesis have been published, are submitted for peer review in journals or are intended to be submitted for publication. This thesis may contain similar passages and/or figures/tables adapted from these manuscripts without distinctly stating their origin, as the attached manuscripts are important part of this thesis. The above said manuscripts have been authored or co-authored by me and were the part of my doctoral thesis work. My contributions to each publication can be found in page 113.

Abstract

Owing to the development of antibiotic resistance in microorganisms, new anti-microbial and anti-infective agents are of dire need to keep these pathogens at bay. These agents are mainly derived from the pathogen (bacteria or fungi) itself or from different plant extracts.

In our previous work, it was observed that Lipoprotein like lipoprotein (Lpl) is an essential virulence factor in *Staphylococcus aureus*. Lpl is membrane bound and encoded in a genomic island called vSaα. Lpl causes the invasion of *S. aureus* into the HaCaT keratinocyte cells through its interaction with Hsp90α receptor. Lpl1 administration also surges the murine kidney abscess - bacterial burden. Our aim was to identify peptides that can block Hsp90 and reduce the *S.aureus* pathogenicity caused by Lpl.

Here, we show 2 small peptides, named L15 and its derivative, L13, both part of Lpl1 have anti-infective action towards *Staphylococcus aureus*. Pretreatment with L15 and L13 reduced the invasion of *S. aureus* USA300 into HaCaT keratinocytes, N/TERT-1 primary keratinocytes and *S. aureus* phagocytosis in human monocytes. The peptides were water soluble, non-cytotoxic and non-hemolytic at the tested concentrations. Dot blot studies showed that there is direct interaction of the peptides with Hsp90α.

L15 and L13 significantly decreased lethality of *S. aureus* bacteremia in *Galleria mellonella* insect larvae model, but did not affect growth or hemolytic activity of *S. aureus in vitro*. In a mouse bacteremia model L15 was found to significantly decrease weight loss and lethality. In effect the small peptides showed the opposite effect from their mother protein, in terms of the invasion of *S. aureus* into host cell and mice bacteremia model.

Although the molecular bases behind the protective effect of L15 remain unclear, *in vitro* data indicate that treatment with L15 or L13 and simultaneous infection with *S. aureus* significantly increase IL-6 production in host immune cells. Our findings highlight Hsp90-interacting peptides as potential anti-infective agents

In a separate work, non-peptide, anti-microbial phytochemicals- Rhodomyrtone (Rom) and Polycyclic polyprenylated acylphloroglucinols (PPAP), capable of inhibiting *S.aureus in vitro* are described. A single point mutation at farR makes *S.aureus*

resistant to Rom. The antibacterial activity of Rom was targeted towards the cell membrane by interacting with its phospatidylglycerol (PG). Rom^R mutants were seen to excrete far more PGs, thereby neutralizing the Rom before it reaches the cell membrane.

PPAP, though very much active *in vitro*, failed to rescue *G. mellonella* larvae from *S.aureus* infection. The reason was found to be the neutralization of PPAPs by the serum albumin proteins found in the larval coelomic fluid. *In silico* docking studies showed that PPAP is binding to the Heme binding pocket of bovine serum albumin. On the other hand, PPAP was observed to have a beneficial but not fully protective effect in *S. aureus* septic arthritis mouse model.

Zusammenfassung

Aufgrund der bei Mikroorganismen steigenden Entwicklung von Antibiotikaresistenzen, werden dringend neue antimikrobielle und antiinfektiöse Wirkstoffe benötigt, um diese Krankheitserreger in Schach zu halten. Diese Substanzen werden hauptsächlich aus Pathogenen (Bakterien oder Pilze) oder aus verschiedenen Pflanzenextrakten gewonnen.

Das *Lipoprotein like lipoprotein* (Lpl), welches in einer genomischen Insel namens vSaα kodiert wird, ist membrangebunden und ein wesentlicher Virulenzfaktor von *Staphylococcus aureus*. Durch die Interaktion von Lpl mit dem Hsp90α-Rezeptor, wird die Invasion von *S. aureus* in HaCaT-Keratinozyten verursacht. Zudem erhöht die Verabreichung von Lpl1, die bakterielle Belastung bei Nierenabszesse in der Maus.

Hier zeigen wir, dass zwei kleine Peptide, L15 und sein Derivat L13, welche beide Bestandteile von Lpl1 sind, eine antiinfektiöse Wirkung auf *Staphylococcus aureus* haben. Die Vorbehandlung der Zellen mit L15 und L13 verringerte die Invasion von *S. aureus* USA300 in HaCaT-Keratinozyten und primären N/TERT-1-Keratinozyten, sowie die Phagozytose von *S. aureus* in menschliche Monozyten. Die Peptide waren in den getesteten Konzentrationen wasserlöslich, nicht zytotoxisch und nicht hämolytisch. Dot-Blot-Studien zeigten, dass es eine direkte Interaktion der Peptide mit Hsp90α gibt.

L15 und L13 verringerten die Letalität von *S. aureus* Bakteriämie im *Galleria mellonella* Insektenlarvenmodell signifikant. Jedoch war weder das Wachstum, noch die hämolytische Aktivität von *S. aureus in vitro* beeinflusst. Anhand eines Bakteriämie Modells in Mäusen wurde festgestellt, dass L15 den Gewichtsverlust und die Sterblichkeit deutlich verringert. Die kleinen Peptide zeigten in Bezug auf die Invasion von *S. aureus* in die Wirtszelle und dem Bakteriämie Modell in Mäusen, die entgegengesetzte Wirkung, verglichen zum Mutterprotein.

Obwohl die molekularen Grundlagen für die schützende Wirkung von L15 noch unklar sind, deuten *in vitro* Daten, auf eine deutlich erhöhte IL-6 Produktion in den Immunzellen des Wirts, nach einer Behandlung mit L15 oder L13 und der gleichzeitigen Infektion mit *S. aureus*, hin. Unsere Ergebnisse weisen auf Hsp90-interagierende Peptide als potenzielle Antiinfektiva hin.

In einer separaten Arbeit werden nicht-peptidische, antimikrobielle Phytochemikalien - Rhodomyrton (Rom) und polyzyklische polyprenylierte Acylphloroglucinole (PPAP) - beschrieben, welche in der Lage sind, *S. aureus in vitro* zu hemmen. Eine einzige Punktmutation in *farR* verursacht in *S. aureus* eine Resistenz gegenüber Rom. Die antibakterielle Aktivität von Rom ist, aufgrund dessen Interaktion mit Phospatidylglycerin (PG), auf die Zellmembran ausgerichtet. Es wurde festgestellt, dass Rom^R Mutanten viel mehr PGs ausscheiden, wodurch Rom neutralisiert wird, bevor es die Zellmembran erreicht.

Obwohl PPAP *in vitro* sehr aktiv war, gelang es nicht, *G. mellonella* Larven vor einer *S. aureus* Infektion zu schützen. Dies resultiert aus der Neutralisierung von PPAPs durch Serumalbuminproteine in der Coelomflüssigkeit der Larven. *In silico docking* Studien zeigten, dass PPAP an die Häm-Bindungstasche von Rinderserumalbumin bindet. Außerdem wurde beobachtet, dass PPAP im Mausmodell, mit einer *S. aureus* verursachten septischen Arthritis, eine positive, aber nicht vollständig schützende Wirkung hat.

Chapter 1

General Introduction

Staphylococcus aureus and its intracellular survival

Staphylococcus aureus is a gram-positive bacterium commonly found on human epithelial surfaces. Nearly 30% of the human population harbors the bacteria on their skin without any signs of infections (Hoter *et al.*, 2018). Sometimes this opportunistic human pathogenic bacterial species can cause grave community acquired and nosocomial infections, comprising wound infection, pneumonia, osteomyelitis, endocarditis, abscess formation, and sepsis/septic shock (Wertheim *et al.*, 2005, Deurenberg & Stobberingh, 2008, Krismer *et al.*, 2017). Management of these infections has become challenging due to the emergence of antibiotic-resistant strains and the capability of *S. aureus* to invade and persist within host cells (Moran *et al.*, 2006, Alva-Murillo *et al.*, 2014).

Lots of studies have shown the capability of *S. aureus* in its invasiveness and persistence within professional phagocytic cells and non-professional phagocytic cells (NPPCs), such as osteoblast, endothelial, fibroblast, epithelial, or kidney cells (Hudson *et al.*, 1995, Vesga *et al.*, 1996, Bayles *et al.*, 1998). For example, Tranchemontagne *et al* showed that *S. aureus* persisted in the macrophages by blocking the development of phagolysosome (Tranchemontagne *et al.*, 2016).

S. aureus owns a collection of virulence factors (i.e. enzymes, invasins, adhesins, toxins) that adds to the pathogenesis of infection, supporting its colonization, dissemination, and transmission. *S. aureus* start the invasion process by attaching to cell membrane of the host cell via the adhesins, like fibronectin-binding proteins A (FnBPA), autolysin (Atl), Extracellular adherence protein (Eap), fibronectin-binding proteins B (FnBPB) and iron-regulated surface determinant B (IsdB) (Sinha *et al.*, 1999, Haggar *et al.*, 2003, Hirschhausen *et al.*, 2010, Zapotoczna *et al.*, 2013). The main method for *S. aureus* internalization into non-professional phagocytes is supposed to be facilitated by FnBPs that network with human $\alpha5\beta1$ integrins via Fn as a bridging molecule. FnBPs also intearct with human Hsp60 thereby ensuring an efficient *S. aureus* internalization into epithelial cells (Dziewanowska *et al.*, 1999, Dziewanowska *et al.*, 2000). This interaction can be direct or indirect, or Hsp60 may work as a co-receptor for $\alpha5\beta1$ integrins (Fowler *et al.*, 2000). The *S. aureus* extracellular adherence protein Eap has been observed to help *S. aureus* Newman

internalization into fibroblasts and epithelial cells. Eap is shown to interact with different plasma proteins, like prothrombin, fibrinogen and fibronectin (Haggar *et al.*, 2003). The autolysins- Atl from *S. aureus* and AtlE from *S. epidermidis* to promote staphylococcal internalization by interacting with the host cell receptor, the heat shock cognate protein Hsc70. The Atl-mediated internalization seems to be the effect of a direct contact between Atl and Hsc70. However, there is an extra interaction of Atl with the host cell integrin $\alpha5\beta1$ with Fn as a bridging molecule. The bacterium adheres to kidney cells, epithelial cells and platelets by employing an iron-regulated surface determinant B protein IsdB, mediated by integrins (Zapotoczna *et al.*, 2013). Lipoproteins (Lpp) are also known to trigger the host cell invasion in epithelial cells (Nguyen *et al.*, 2015). This bridging of bacteria and host cell proteins triggers bacterial uptake by mechanism driven by the host cells that involves actin remodeling, Src family kinases and focal adhesion kinase (Agerer *et al.*, 2005, Sinha & Herrmann, 2005).

Lipoproteins

The first Lipoprotein (Lpp) elucidated was from *E. coli* and is called Braun's Lipoprotein (Hantke & Braun, 1973). Lpp is attached with its N-terminal lipid structure in the outer membrane of nearly all Gram-negative bacteria. In Gram+ bacteria, the Lpp's lipid moieties are attached in the outer layer of the cytoplasmic membrane and their protein portions extent to the cell wall.

Biosynthesis of Lipoproteins

Lipid modification enables the binding of hydrophilic proteins in bacteria to the hydrophobic cell wall phospholipids. Here in lipoproteins, an acyl moiety is attached to the protein, which provides a membrane anchor for its easy function. Lipoproteins are synthesized in the cytoplasm as precursors and are then translocated across the cytoplasmic membrane. These precursors possess an N-terminal signal peptide with a characteristic "lipobox" consensus region at its C-terminal. In the lipobox sequence: Leu-(Ala/Ser)-(Gly/Ala)-Cys, the last cysteine is modified by covalent attachment of a diacylglycerol moiety to its thiol group by the enzyme lipoprotein diacylglyceryl transferase (Lgt). Following lipid modification, the signal sequence of the lipidated prolipoprotein is cleaved just before the cysteine residue by lipoprotein signal

peptidase (Lsp). The leaves the cysteine of the Lipobox as the new N-terminal residue of the lipoprotein or the mature lipoprotein in Gram-positive bacteria.

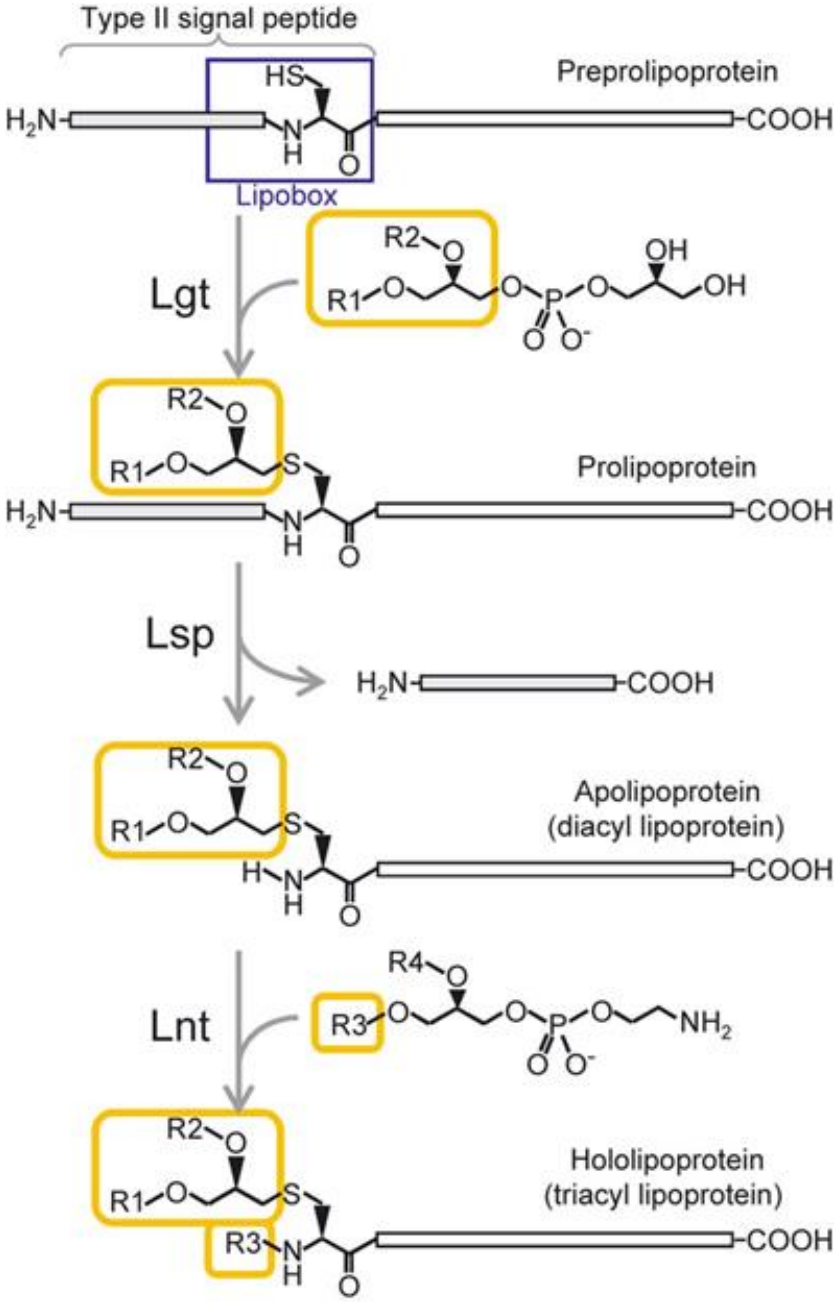


Figure 1.1. Biosynthesis of lipoprotein in bacteria. Adapted with Rightslink permission from (Nakayama *et al.*, 2012)

In gram negative bacteria, the Lsp catalyzed lipoprotein is further modified at the N-terminal cysteine group by acylation reaction (Addition of an amide – linked acyl group). This step is catalyzed by lipoprotein N-acyl transferase (Lnt) (Kovacs-Simon *et al.*, 2011) (Figure. 1.1). Both the membrane phospholipids-derived amino-terminal acyl groups (in gram –ve bacteria) and the diacylglyceryl group contributes to tight

anchorage of the lipoprotein to the membrane (Hantke & Braun, 1973) . The enzymes involved in the biosynthesis of lipoproteins have been employed as a target for the synthesis of new antibacterial agents. This is because of the nonexistence of any homologues in eukaryotes.

Functions of bacterial lipoproteins

Bacterial lipoproteins have different functions varying from virulence in pathogenic bacteria to nutrient uptake signal transduction etc. The functions of bacterial lipoproteins are given in detail below. Nguyen et al., compared the Lpp of 14 bacterial species from the genera of *Bacillus, Clostridium, Enterococcus, Listeria, Mycobacterium, Staphylococcus and Streptococcus.* The number of Lpp genes in each of the 14 mentioned species are different but accounts for a substantial part of the genome (1-3 %) (Nguyen *et al.*, 2020).

Functions of Lpps are

1. Transportation: *S. aureus* USA300 (MRSA) has 67 Lpps, in which nearly 1/3rd are involved in transportation of zinc (Zn),iron (Fe), manganese (Mn), Nickel (Ni), molybdenum (Mo), Cobalt (Co), aminoacids, sugars etc. Different Lpps involved in the transport are mentioned below.

- Iron acquisition: *S.aureus* has 8 widely iron transporting Lpps which include FhuD1, FhuD2 (both bind to iron(lll)-hydroxamate siderophores and present it to the ABC transporter – FhuCBG), SirABC (transport of staphylobactin, ferric enterobactin or ferric citrate and ferric hydroxamates) and FepA (part of FepABC - containing FepA (Fe binding Lpp), FepB (with TAT signal peptide) and FepC (transport of Fe across the memrane)) (Sebulsky *et al.*, 2000, Dale *et al.*, 2004, Biswas *et al.*, 2009). The pathogenicity of staphylococci was found to be linked directly to lipoprotein dependent Fe-uptake systems (Shahmirzadi *et al.*, 2016). In *S.pyogenes*, both MtsA (part of MtsABS transporter) and SiaA (part of SiaABC transporter) are Lpp involved in Fe acquisition (Sun *et al.*, 2008, Sun *et al.*, 2010).
- Other cation and anion transporters: the Lpp MntC in *S.aureus* is involved in the transport of Mn. Homologues of MntC were found in bacilli as MntA and streptococci as PsaA (in *Streptococcus agalactiae* and *Streptococcus*

pneumoniae). 3 Lpp in *S.aureus* were glossed as Nickel transporters. Other Lpps functioning as cationic transporters include Cnt (Opp1) (Ni and Co transport at zinc reduced conditions in *S.aureus*), ModA (involved in Mo transport), AdcA and AdcAII (zinc transport in *Streptococcus*) (Neubauer *et al.*, 1999, Remy *et al.*, 2013, Tedde *et al.*, 2016). In USA300, the annotated Lpps - USA300_1283, USA300_0145 and USA300_0175 are involved in the transport of phosphate, phosphonate and nitrate respectively (Shahmirzadi *et al.*, 2016).

- Amino acid transport: *S.aureus* has 7 identified Lpps involved in the transport of aminoacids and short peptides. These includes Opp3 (involved intransport of oligopeptides) and GmpC (for transport of glycyl - methionine) (Williams *et al.*, 2004, Hiron *et al.*, 2007). Other Lpps functioning as amino acid/peptide transporters are OppA (from OppABCD(F) system), AppA (from AppDFABC transport system) and MetQ (from MetQNP transporter) (Koide & Hoch, 1994). MetQ trasports methionine in streptococci and is found to be significant in its virulence and growth function (Basavanna *et al.*, 2013).
- Sugar and lipid transport: The only Lpp involved in the sugar transport is the maltose ABC transporter (Sauvageot *et al.*, 2017). The Mce Lpps in *Mycobacterium tuberculosis* transports cholesterol through the cell envelope. The Mce is crucial in the host signalling and the bacterial growth with cholesterol as the only carbon source (Wilburn *et al.*, 2018).

2. Cell wall biosynthesis and degradation: Studies about the role of Lpps in cell wall biosynthesis and degradation are very few. A papain-like cysteine peptidase called New Lipoprotein C/Protein of 60-kDa (NlpC/P60) is said to catalyse D-γ-glutamyl-meso-diaminopimelate or N-acetylmuramate-L-alanine linkages (Anantharaman & Aravind, 2003, Vermassen *et al.*, 2019). Lpps functioning as penicillin binding proteins, cell elongation specific DD-transpeptidase, and polysaccharide deacetylase were seen in some Bacilli. The lytA Lpp in Bacillus group, is a part of the operon lytRABC, which regulates the N-acetylmuramoyl-L-alanine amidase gene (Lazarevic *et al.*, 1992).
3. As foldases and Enzymes:

- YidC first found in *E.coli* is a membrane insertase Lpp that helps in the assembly and insertion of various proteins in the bacterial membrane (Samuelson *et al.*, 2000).

- PrsA or Peptidylprolyl Isomerase, a membrane bound Lpp is crucial in protein synthesis in *Bacillus subtilis* (Kontinen & Sarvas, 1993). PrsA is also known to fast-track the folding of proteins encompassing cis- proline (Gothel & Marahiel, 1999).
- SCO or Synthesis of Cytochrome c Oxidase (in Bacillu species) and its homologues (QoxA in *S. aureus*, Etrx1 and Etrx2 in *S. pneumonia*) are essential in the respiratory chain and sometimes during oxidative stress (Gotz & Mayer, 2013, Saleh *et al.*, 2013, Xu *et al.*, 2015).
- Lpp-penicillinases are found in both membrane bound and secreted free form with their function as β-lactamases in many bacterial species (Nielsen & Lampen, 1982). Lpps are also known to have protease, phosphatase, peptidase, esterase or lipase functions. The Lpp in *Streptococcus equisimilis,* called LppC does the role of as an acid phosphatase (Wolschendorf *et al.*, 2007).
- LanI or Lantibiotic Immunity Lpp in gram positive species makes the pathogen resistant to nisin specifically (Hacker *et al.*, 2015, Geiger *et al.*, 2019).
- Sex pheromones: Derivatives of signal sequences from the C-terminals of Lpp are employed as sex hormones in some bacterial species including *S. aureus* (cAM373)*, S. epidermidis, E. faecalis* (cAM373)*, L. monocytogens* and *B. subtilis*. These hormones expedite the conjugative transfer of certain plasmids in these bacteria (Dunny & Leonard, 1997, Flannagan & Clewell, 2002).

The information about many Lpps in terms of their role and importance is still incomplete. Although Lpps are seen in nearly every bacterial species, and is conserved in many, each Lpp is unique subject to the habitat and genetic makeup of the bacteria.

Lpl, A Special Class of Lpp in *S.aureus,* contributes to Invasion and virulence

Most *S. aureus* strains contain a pathogenicity island, *vSaα*, which encodes a set of lipoprotein-like gene cluster called *lpl* (lipoprotein like lipoprotein). Particularly, in highly epidemic strains the *lpl* cluster comprises up to 10 homologous tandemly arranged *lpl* genes (Nguyen *et al.*, 2015). It has been found that the *lpl* gene cluster contributes to internalization into NPPCs such as keratinocytes and epithelial cells. Here, USA300 wt, USA300 ΔlpI mutant and the respective complemented mutant were checked for its invasion capacity differentiated primary keratinocyte cells. In cells infected with ΔlpI

mutant, the number of invaded bacteria were 2.5 fold lower compared to the wild type USA300 strain.

On complementation of the same gene, the intracellular bacterial count went up to 1.5 in comparison to the wild type, suggesting the significance of *lpl* gene cluster in invasion in keratinocytes. *S. carnosus*, a relatively non-pathogenic bacteria doesn't have a lipoprotein cluster. A mutant of *S. carnosus* which expresses the Lpl - (*S. carnosus* (pTX30::lpl)) showed higher invasion potential as compared to *S. carnosus* wt and *S. carnosus* (pTX30). This result reinforced the significance of *lpl* gene cluster in invasion in keratinocytes. Similar trend was also seen on HeLa cells. They could also observe a cell cycle delay (G2-M transition) in HeLa cells on exposure to Lpl proteins.

The importance of *lpl* gene cluster in virulence were also seen *in vivo*. On challenging of Balb/C mice with USA300 wt and USA300 Δlpl mutant, Nguyen *et al.*, found that, the bacterial burden in the kidney of Δlpl mutant group were significantly lower than the USA300 wt infected group (Nguyen *et al.*, 2015). Lpl1 has been used as a model protein in the mice experiments.

Mohammad M *et al*., injected Lpl1 into mice knee joint intra-articularly and saw macroscopic inflammation at the joints after 24hrs (Mohammad *et al.*, 2019). They could also observe synovitis and severe bone erosions indicating an *S. aureus* septic arthritis. The Lpl1 without the signal peptide called Lpl1 (-sp), failed to induce arthritis suggesting that the lipid moiety of Lpp is responsible for the arthritic induction. It was also observed that the TLR2 is required for the monocyte and neutrophil migration to synovial site after the Lpl1 administration.

Lpl protein triggers host cell invasion via activation of Hsp90 receptor

Recently it has been found that Lpl1 triggers the invasion into HaCaT cells by activating the Hsp90α of the latter (Tribelli *et al.*, 2020). Hsp90 is a chaperone proteins and are highly expressed in most of the eukaryotic cells. Because of its involvement in the stabilization of lots of oncogenic proteins, Hsp90 inhibitors are widely studies as cancer treatment drugs. Hsp90α is said to be membrane bound, but is also found as secreted

form in response to tissue injury (Guo *et al.*, 2017). Blocking of the keratinocyte Hsp90 using antibodies resulted in reduced USA300 invasion and adherence into the host cells. The reduction in USA300 invasion to Hsp90α silenced HaCaT cells added to strengthening the above said theory. Lpl1-Hsp90 interaction induces F-actin formation, thus, triggering an endocytosis-like internalization (Figure 1.2). Even though there is direct interaction with Lpl1 and Hsp90, Tribelli *et al*., couldn't see any resultant effect on the ATPase activity of Hsp90.

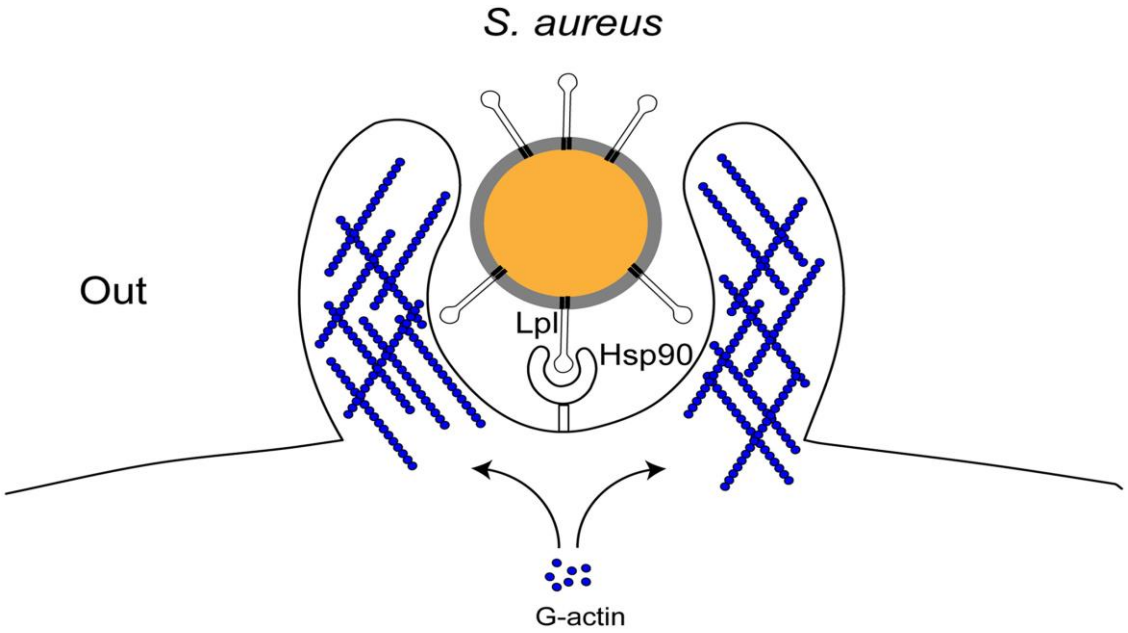


Figure 1.2. Proposed model of Lpl-Hsp90 interaction during USA300 invasion in keratinocyte cell. The C-terminal region of Lpl1 interacts with Hsp90. This interaction triggers F-actin formation and the bacteria is taken in by an endocytosis like process. G-actin: monomer of actin protein, F-actin: filamentous actin. Adapted from (Tribelli *et al.*, 2020).

Galleria mellonella larvae as an infection model

Galleria mellonella (order *Lepidoptera*, family *Pyralidae*) also known as the greater wax moth or honeycomb moth has been widely studied as an infection model to study microbial pathogenesis and efficacy of different drugs. *G. mellonella* larvae are cheaper to acquire and can be easily maintained in large numbers. The development of *G. mellonella* infection model is simple and doesn't require much high end lab equipment. The lack of ethical constraints in *G. mellonella* study and their short life cycle makes them ideal candidate for these studies. The survival of *G.*

mellonella larvae at 37°C makes them more preferable in comparison to other commonly employed invertebrate models such as *Drosophila melanogaster* and *Caenorhabditis elegans* (Tsai *et al.*, 2016). Although the greater wax moth lack an adaptive immune response, their innate immune response shows remarkable similarities with the immune response in vertebrates. The innate immune system includes both humoral and cellular immune response. The humoral immune reaction is mediated by effector molecules that are able to kill or trap the pathogen. These effectors can be opsonins or antimicrobial peptides (AMPs).

Opsonins in *G. mellonella* include peptidoglycan recognition proteins (PGRPs), apolipophorin III (apoL-III), cationic protein 8 (GmCP8) and hemolins (Seitz *et al.*, 2003). PGRPs binds to the peptidoglycan of bacteria and were identified among one of the genes induced by LPS in the hemocyte (Seitz *et al.*, 2003, Dziarski & Gupta, 2006). Apolipophorin III, a pattern recognition molecule binds to hydrophobic ligands - lipopolysaccharides and lipoteichoic acids, activating the innate immune response (Halwani *et al.*, 2000, Pratt & Weers, 2004). GmCP8 work as a multi ligand protein in the phagocytosis of pathogens (Kim *et al.*, 2010). Hemolin are expressed in the silk glands and nervous system of larvae. Hemolin, generally upregulated when triggered by β-glucans, marks the apoptic/infected cells for elimination by hemocytes (Shaik & Sehnal, 2009, Mowlds *et al.*, 2010).

The greater wax moth have a wide array of anti-microbial peptides which includes cecropin, lysozyme, moricin like peptides, galliomycin, gloverin, defensin, gallerimycin, apolipophoricin and x-tox. In addition G. mellonella's AMPs also include Gm proline-rich peptides 1 and 2, inducible serine protease inhibitor 2, heliocin-like peptide and Gm anionic peptide 1 and 2 (Cytrynska *et al.*, 2007, Brown *et al.*, 2009). Lysozymes catalyzes the hydrolysis of the β-1, 4 linkage between N-acetylglucosamine and N-acetylmuramic acid in the cell wall peptidoglycan (Sowa-Jasilek *et al.*, 2014). It was observed that the synergistic action of apoL-III and lysozyme increases the permeability of Gram-negative bacteria (Zdybicka-Barabas *et al.*, 2013). Cecropins and moricins (α-helical peptides) and defensins (cationic peptides) causes ion leakage and subsequent cell lysis in both Gram + and –ve bacteria by forming pores in the cell walls and membranes (Kim *et al.*, 2004, Brown *et al.*, 2008). Studies have shown that the activity of cecropin is sometimes enhanced by apoL-III. *G. mellonella* also have small peptides of size ranging from 2-4 kDa, called the proline-rich peptides that can

enhance the permeability of bacterial membrane and are known to inhibit the growth of yeast (Cytrynska *et al.*, 2007). Another AMP, gloverin binds to LPS of the Gram-ve bacteria and causes membrane permeability by inhibiting the synthesis of some vital outer membrane proteins (Kawaoka *et al.*, 2008). Other AMPs include gallerimycin, that can inhibit fungi and X-tox, whose function is still unknown (Langen *et al.*, 2006).

Plant derived antimicrobials

The abuse or overuse of antibiotics for every human ailment has resulted in antibiotic resistance in pathogens (Yu *et al.*, 2020). Antibiotic resistance is one of the prominent reason for increased mortality and morbidity in patients with infections, and this has caused a giant economic burden on out healthcare system (The Lancet Infectious, 2017). In 2017, World Health Organization (WHO) published its first list of pathogens that are antibiotic resistant, and are a threat to the population's health. This list covers12 bacterial families, for which new antibiotics against them are of dire need. *Staphylococcus aureus* is included in this 12 family list, as a high propriety organism (Khare *et al.*, 2021). The Centers for Disease Control and Prevention (CDC) and the European Centre for Disease Prevention and Control (ECDC), classify pathogens into multidrug resistant (MDR), extensively drug resistant (XDR) or pandrug resistant (PDR) based on their resistance to antibiotics. Pathogens resistant towards more than one class of antibiotics are called Multi drug resistant pathogens.

Secondary metabolites from different plants exhibit promises in its ability to kill these resistant pathogens. Many plant extracts (in crude or modified form) have been employed in cosmetics and food industry to prevent product spoilage. Phytochemicals such as capsaicin, colchicine, paclitaxel, and reserpine has been approved by the Food and Drug Administration (FDA), and are effective against many MDR pathogens. The mode of action of these phytochemicals were mainly found to be- by inhibiting bacterial replications of the efflux pumps or by increasing the bacterial cell permeability to anti-microbial.

Phytochemicals can be essential oils, alkaloids, flavonoids or phenolic compounds. For example, essential oils extracted from the barks of cinnamon has been proved to be strongly bactericidal against MDR *S.aureus* (Naveed *et al.*, 2013). Piperine, an alkaloid from *Piper nigrum* is known for its activity against MRSA by the inhibition of the bacterial efflux pump (Khan *et al.*, 2006, Khameneh *et al.*, 2015). Catechin, an

antibacterial flavonoid mainly seen in tea and legumes are known to attack the methicillin-resistant *Staphylococcus aureus* (MRSA) membrane, resulting in potassium leakage (Cushnie *et al.*, 2008). Another phenolic-acid phytochemical, gallic acid is observed to have solid antibacterial activity against *S. aureus, S. agalatiae, P. aeruginosa, E. fecalis, E. coli* etc. (Cueva *et al.*, 2012). Although there are several prosperous examples of phytochemicals against antibiotic resistant infections, most of them fail to reach the commercial pharmaceutical market and this needs to be remedied.

Rhodomyrtone

Rom or rhodomrytone, an acylphloroglucinol isolated from *Rhodomyrtus tomentosa* is found to be active against different gram +ve bacteria like *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Propionibacterium acnes* and multidrug-resistant *Enterococcus faecalis* (Voravuthikunchai *et al.*, 2010, Limsuwan *et al.*, 2011, Saising & Voravuthikunchai, 2012, Wunnoo *et al.*, 2021). Rom's anti-microbial action is directed towards the bacterial cell membrane. The binding of Rom to the phosphatidylglycerol head of the phospholipid membrane causes the membrane to fold on to itself and subsequently its disruption. This disruption of cell membrane results in the leakage of ATP and different cytoplasmic proteins thereby bringing down the membrane potential (Saising *et al.*, 2018). Rom was observed to reduce the FtsZ polymerization and inhibit GTPase activity in *Bacillus subtilis*. FtsZ is a protein that drives the cell division in bacteria and is a homolog of tubulin in eukaryotes. FtsZ polymerized to form a Z-ring, causing the cell wall and membrane to constrict and form septum, resulting in the creation of 2 daughter cells. FtsZ is crucial in every bacteria and the polymerization requires GTP (Saeloh *et al.*, 2017).

Nyugen et al., were able to develop Rom resistant *S.aureus* HG001 mutant strains by continuous subculture of the wild type strain in Rom supplemented media. The whole genome sequencing of the resistant mutant enabled them to identify a specific single point mutation instigating the Rom resistance in HG001. This point mutation was a change in the codon TGC to CGC resulting in change in the amino acid from cysteine (Cys) to arginine (Arg) in the coding region of *farR* (regulator of fatty acid (FA) resistance) at position 116 (Nguyen *et al.*, 2019). *FarR* expresses a regulator which controls *farE.* FarE is a transmembrane protein which functions as an efflux pump for multiple drugs (Alnaseri *et al.*, 2015). The point mutation in farR (*farR**) inactivates the

repressor function. This leads to an up-regulation of *farE*, thereby increasing the resistance of the strains against FA antimicrobials. More FarE means more efflux of FAs from bacterial cells. Nyugen et al., could also observe that the genes *psmβ1* and *hla*, encoding toxins and *sspB* and *gehA*, encoding secreted enzymes were also upregulated in Rom^R strain, resulting in enhanced virulence of the mutant in mouse pneumonia model. (Nguyen *et al.*, 2019) (Figure 1.3B).

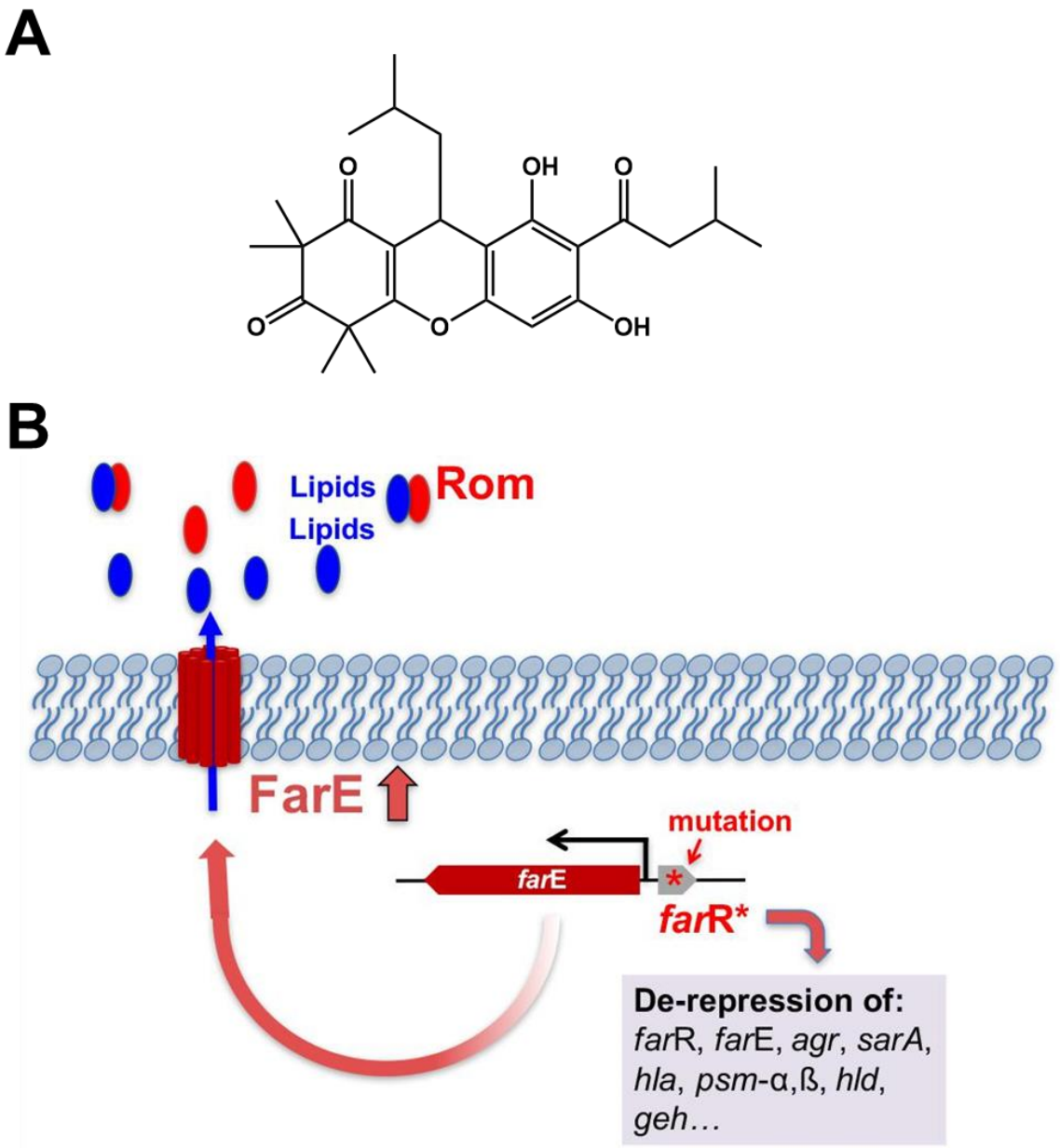


Figure 1.3. (A) Structure of Rom drawn using ChemDraw Professional 16.0. (B) Mechanism of Rom resistance in Rom^R mutant facilitated by the FarE. Reprinted under the Creative Commons Attribution License (CC BY) from (Nguyen *et al.*, 2019).

Polycyclic polyprenylated acylphloroglucinols (PPAPs)

Polycyclic polyprenylated acylphloroglucinols (PPAPs) from *Hypericum perforatum* or St. John's wort, is a well-known antidepressant and its derivatives are active against different microbes (Barnes *et al.*, 2001). PPAP features a bicycle [3.3.1]nonane-2,4,9-trione and can be classified into 3 types depending on the position of the exocyclic acyl group. They are- type A with acyl group at C1, type B with acyl group at C3, and type C with acyl group at C5 position (Figure 1.4). Hyperforin, the first PPAP isolated, is type A PPAP used for the treatment of depression, schizophrenia and anxiety (Ciochina & Grossman, 2006).

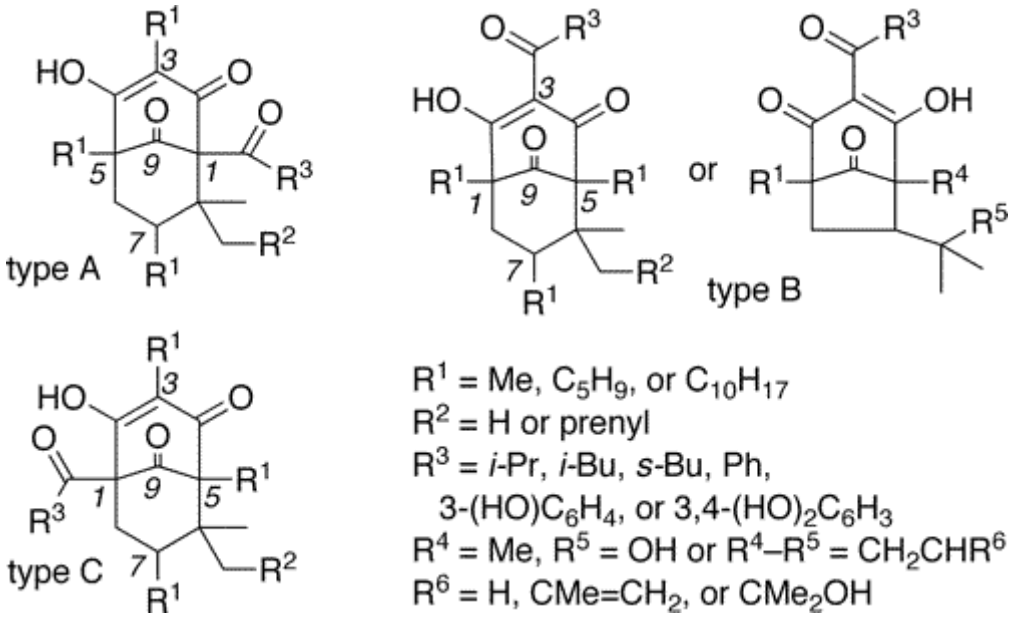


Figure 1.4. Type A, B and C PPAPs. Adapted with permission from (Ciochina & Grossman, 2006). Copyright 2022 American Chemical Society.

Wang et al., synthesized a type B PPAP called PPAP23, which was found to be active against different gram positive bacterial strains. PPAP23 showed an MIC of 1ug/ml same as that of vancomycin against *S. aureus* USA300. The compound was also found to be active against Staphylococcus aureus Mu50, a VISA strain, *Enterococcus faecium* 4147, vancomycin-resistant *Enterococcus faecalis* VRE366, *Enterococcus faecium* VRE392, as well as *Streptococcus pneumoniae* ATCC49619 and *Listeria monocytogenes* ATCC19118 with MIC values < 5 µg/mL (Guttroff *et al.*, 2017). Wang et al., didn't observe any membrane depolarization or pore formation in *S. aureus* USA300 upon PPAP23 treatment, but noticed extensive ATP leakage and reduced consumption of oxygen in the bacteria. This suggested that PPAP targets the bacterial

membrane and its respiration (Figure 1.5). The exposure to PPAP also caused in the loss of bacterial resistance to osmotic pressure. PPAP was also seen to hinder with the bacterial iron metabolism. Genes involved in iron acquisition were downregulated and the one involved in iron storage was upregulated. PPAP23 also caused ROS production in the bacterial cell (Wang *et al.*, 2019).

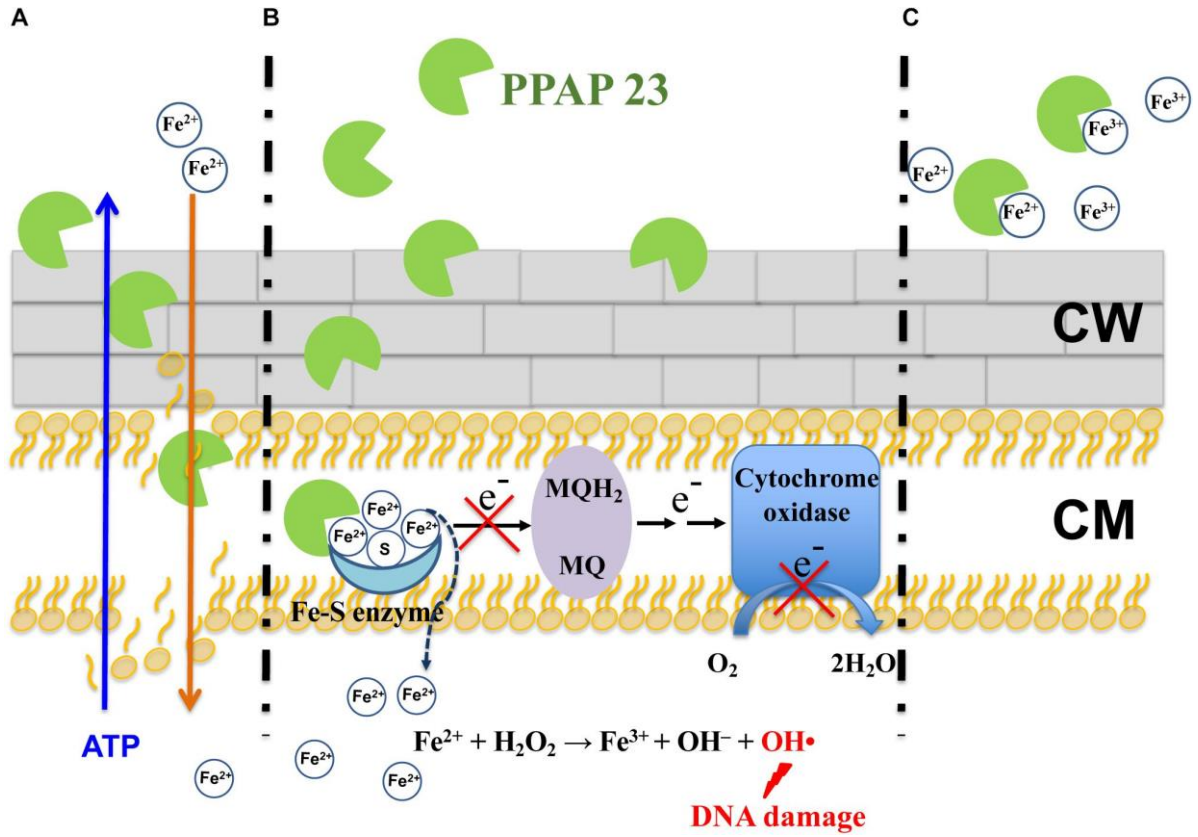


Figure 1.5. Proposed antibacterial action of PPAP23. (A) PPAP 23 affects the membrane integrity by interacting with the lipophilic pocket of the membrane. Disintegrated membrane results in the diffusing out of ATP and diffusing in of iron to the bacterial cells. (B) PPAP 23 chelates iron from Fe–S cluster enzymes causing the inactivation of Fe–S cluster enzyme. This can disturb the cell respiration (through the enzymes involved) or DNA damage and cell death (due to iron overload). (C) PPAP 23's antimicrobial activity is lessened when it is iron bound. MQH2, menaquinol; MQ, menaquinone; CW, cell wall; CM, cytoplasmic membrane. Adapted with permission from (Wang *et al.*, 2019) with Creative Commons public licenses.

Objectives

This work is a continuation of the previous work where Hsp90α was recognized as the host cell receptor for Lpl in USA300. The potential of Lpl1 fragments of different sizes were analyzed for their influence in USA300 invasion into HaCaT cells. A 15 amino acid fragment of Lpl1 called L15 (NH2-TAKGHYFVTTFYRNG-COOH) and its derivative L13 (NH2-GHYFVTTFY-COOH) were seen to bind directly to the Hsp90α, but unlike Lpl1, reduces the invasion of bacteria into the keratinocytes. This intrigued us to check its potential on the invasion of USA300 on primary keratinocytes (N/TERT-1) and monocytes. The cytotoxic effect of these peptides on different eukaryotic cells and their hemolytic activities were also tested.

Next, we checked if the peptides can rescue *Galleria mellonella* larvae from different strains of S.aureus infection. The efficacy of the peptides in rescuing the same larvae from infection caused by bacteria including *E. coli, P. aeruginosa* were also studied. Growth curve kinetics study and hemolytic activity of S.aureus on treatment with L15 and L13 were conducted to identify if the larval rescual is the result of the peptides compromising the growth or virulence of the bacteria. Once it was seen that the peptides indeed could protect the larvae from *S.aureus* bacteremia, the same study was replicated in mice model with L15 as the model peptide.

Next, immune stimulation experiments were carried out to see the effect of L15 on PBMC cells alone and also in combination with USA300 infection. Multiple sequence alignment of lipoprotein analogs in different bacteria as well as similarity between different Lpls (Lpl1 - 9) were examined. Additionally, the potential of the peptides in rescual of larvae from S. aureus bacteremia in combination with different TLR ligands were also analyzed.

The group of Martin Maier, Institut für Organische Chemie, Universität Tübingen, was not only able to chemically synthesize Rom but also its isomer, rhodomyrtosone B; both isomers showed similar high antimicrobial activity. Despite its antibacterial activity on a broad spectrum of bacteria, the complete mode of action of Rom is still unknown other than the observation that Rom targets the cell membrane (Saising *et al.*, 2018).

The objective of this paper is to cement our previous *in silico* data and hypothesis that Rom binds to the phospatidylglycerol (PG) group of the cell membrane by employing lipidomic analysis *in vitro* and to understand further about the molecular mechanism of Rom resistance. More Rom derivatives were also synthesized to develop a Rom derivative molecule that is also active against the Rom^R strain. The solubility of Rom derivatives was another criterion during the derivative synthesis. Most Rom derivatives are insoluble in water and are soluble only in DMSO. The activity of Rom against different anaerobic strains were also tested.

To assess the role of *farE* in Rom resistance, the said gene was deleted in *S.aureus* HG001 and the corresponding Rom^R strain and was checked for its sensitivity to rhodomyrtone. Next, a lipidomic analysis of the culture supernatants was carried out to check whether the cause of Rom resistance was due to (1) Expulsion of Rom by the FarE pump or (2) Neutralization of Rom by the excreted PGs. Static and dynamic light scattering and Isothermal titration calorimetry experiments were carried out to check the interaction of Rom with PG. In static & dynamic light scattering studies, one can measure the diameter of Rom and PG vesicles separately and this diameter will increase if there is an interaction. The effect of another membrane protein MprF on Rom resistance was also investigated. MprF synthesizes Lys-PG and rescues the bacteria from lipopeptide antibiotics and cationic anitimicrobial peptides. Additionally, we tested whether resistance to Rom can lead to cross-resistance in other antibiotics.

PPAP23 was found to be active gainst *S.aureus* and other anaerobes including *Clostridium difficile, Clostridium perfringens, Streptococcus salivarius, Ruminococcus gnavus, Clostridium ramosum, Blautia obeum* and *Parabacteroides distasonis*. A new water-soluble derivative of PPAP23, called PPAP53 was synthesized. Both PPAP23 and PPAP53 have similar MICs (0.5 -1 µg/ml) against *S.aureus*. Despite its promising antibacterial activity *in vitro*, both PPAP23 and PPAP53 were unsuccessful in providing a protecting effect on *S.aureus* infected *Galleria mellonella* larvae upon treatment. The control vancomycin protected 100% larvae from infective death. The objective of this paper is to understand the reason why PPAP fails to rescue the larvae from infection and identify the compound (serum albumin) in larval coelomic fluid responsible for its neutralization.

Once identified, further *in vitro* bacterial growth curve kinetics will be carried out to understand the bactericidal effect PPAP in combination with neutralization molecule - serum albumin. *In silico* docking studies will also be carried to identify the site of interaction of PPAP and the neutralization molecule (serum albumin). Further growth kinetics experiments involve blocking different sites of serum albumin with its ligands and then testing the efficacy of PPAP in killing *S. aureus* USA300 in the presence of the ligand saturated albumin. The cytotoxicity of PPAP23 (and some derivatives) in presence and absence of albumin proteins were also tested using MTT assay. The effect of PPAP23 on mice septic arthritis were also investigated. This is done by comparing the severity of clinical arthritis, the body weight development, Kidney abscess scores and quantity of *S. aureus* in kidneys of infected mice and that of infected mice treated with PPAP.

Chapter 2

Materials and Methods

Materials

Chemicals and media

Chemicals and media	Manufacturer
Acetic acid	Sigma-Aldrich
ActinGreen™ 488 ReadyProbes®	Thermo Fischer, Germany
alkaline phosphatase conjugated goat-α-mouse IgG	Sigma-Aldrich, Germany
Ampicillin	AppliChem GmbH, Darmstadt, Germany
Anti-Hsp90 antibody	Abcam, Germany
anti-human CD14 microbeads	Miltenyi Biotech, Bergisch-Gladbach, Germany
Bovine serum albumin	Sigma-Aldrich Chemie Gmbh, Munich, Germany
CD14-FITC	BD Biosciences, Heidelberg, Germany
CD45-PE	BD Biosciences, Heidelberg, Germany
DMEM, high glucose, with phenol red	Thermo Fisher Scientific, Schwerte, Germany
DMSO ≥99 %	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
DNAse I	Applichem GmbH, Darmstadt, Germany
Ethanol	Sigma-Aldrich
Ethylene diamine tetraacetic acid (EDTA)	Applichem GmbH, Darmstadt, Germany
Ethylene diamine tetraacetic acid (EDTA)	Applichem GmbH, Darmstadt, Germany
Geldanamcin	Sigma-Aldrich Chemie Gmbh, Munich, Germany
Gibco Roswell Park Memorial Institute 1640 Medium (RPMI 1640)	Thermo Fisher, Waltham, MA, USA

Glucose D(+)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Hydrochloric Acid	Sigma-Aldrich Chemie Gmbh, Munich, Germany
Isopropanol	Sigma-Aldrich Chemie Gmbh, Munich, Germany
Kanamycin	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Keratinocyte serum-free medium (K-SFM)	Gibco, Invitrogen Corp.
LB media	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
L-Glutamine	Thermo Fisher Scientific, Schwerte, Germany
Magnesium chloride	Sigma-Aldrich Chemie Gmbh, Munich, Germany
Milk powder, blotting grade	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Penicillin Streptomycin	Thermo Fisher Scientific, Schwerte, Germany
Phosphate Buffered Saline (PBS),1x	Thermo Fisher Scientific, Schwerte, Germany
Pierce™ BCA Protein Assay Kit	Thermo Fisher Scientific, Schwerte, Germany
Propidium iodide	BD Biosciences, Heidelberg, 436 Germany
Recombinant Hsp90α	Thermo Fisher Scientific, Schwerte, Germany
Roti®-Quant, 5X concentrate	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium chloride	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium dodecyl sulfate (SDS)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany

Triethylamine (TEA)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Triton X-100	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Gibco Trypsin-EDTA (0.25%), with phenol red	Thermo Fisher, Waltham, MA, USA

Consumables/ Standard labware

Consumables	**Manufacturer**
0.2 µ filter tips	Sarstedt
0.45 µ filter tips	Sarstedt
10 mL pipette	Greiner
12 well plate Cell culture	Greiner
24 well plates Cell culture	Greiner
25 mL pipette	Greiner
48 well plate (Growth curve)	Greiner
48 well plates Cell culture	Greiner
6 well plates Cell culture	Greiner
96 well F bottom	Greiner
96 well lid with CR	Greiner
96 well plates Cell culture	Greiner
96 well U bottom	Greiner
96 well U bottom black plates	Greiner
Cell Scrapper	Cornig
Cryopreservation tubes	Greiner
Cuvette	Sarstedt
ELISA plates	Cornig Sigma
Eppendorfs 1.5 mL	Fischer scientific
Eppendorfs 2 mL	Fischer scientific
Eppendorfs 5 mL	Fischer scientific
Falcon tube 15 mL	Greiner
Flacon tube 50 mL	Greiner

Gloves	Haeberle
Inoculation loop 10 uL	Sarstedt
Inoculation loop 1uL	Sarstedt
Pipette tips 10 µL	Axygene from sigma
Pipette tips 100 µL	Greiner
Pipette tips 1000 µL	Greiner
PP tube 14ml	Greiner
RAININ pipette tips 1200 µL	Mettler Toledo
RAININ pipette tips 200 µL	Mettler Toledo
Reservoir	Fischer Scientific
Square Petri plates	Fischer scientific
Syringe 10 mL	Haeberle
Syringe 20 mL	Haeberle
Syringe 5 mL	Haeberle
T spreader	TH Geyer
T-25 Cell culture flask	Greiner
T-75 Cell culture flask	Greiner

Technical equipment

Equipment	**Manufacturer**
Centrifuge 5424R	Eppendorf AG, Hamburg, Germany
Centrifuge 5810R	Eppendorf AG, Hamburg, Germany
CO2 Incubator, BBD 6220	Heraeus Instruments
Flow cytometer BD Accuri C6	BD Biosciences, Heidelberg, Germany
Laminar flow hood	Tecnoflow, Cesate MI, Italy
Magnet stirrer RCT basic	IKA Werke GmbH, Staufen, Germany
Microscope, Zeiss Axioplan 2	Zeiss, Oberkochen, Germany
Multichannel pipette	Eppendorf AG, Hamburg, Germany
pH meter	Mettler Toledo GmbH, Giessen, Germany
Pipetts	Brand GmbH & Co KG, Wertheim, Germany
Pipettboy	Integra BioSciences, Fernwald, Germany
Refrigerator	Liebherr, Germany

Spectrometer NanoDrop 2000	Thermo Scientific, Schwerte, Germany
Sterile hood	BDK GmbH, Sonnenbühl-Genkingen, Germany
Varioskan LUX multimode microplate reader	Thermo Scientific, Schwerte, Germany
Vortex Genie 2	Scientific Industries, Karlsruhe, Germany
Water bath	GFL Technology, Lauda-Koenigshofen, Germany

Mammalian cell lines

Name	**Type Of Cell Line**	**Source**
$CD14^+$ Monocytes	$CD14^+$ Monocytes	German Red Cross Blood transfusion west (Hagen, Germany)
HaCaT	Immortalized Keratinocyte Cells	German Cancer Research Center (DKFZ)
HEK	Human Embryonic Kidney Cells	ATCC
HL-60	Acute Monocytic Leukemia	German Collection of Microorganisms and Cell Cultures (DSMZ)
HT-29	Human Colon Cancer Cells	ATCC
MM6	MONO MAC 6, Acute Monocytic Leukemia	German Collection of Microorganisms and Cell Cultures (DSMZ)
N/TERT-1	Immortal Keratinocyte Cell Line	Dr. J. Rheinwald, Harvard Medical School, Boston, USA
PBMC	Peripheral Mononuclear Blood Cells	German Red Cross Blood transfusion west (Hagen, Germany) and the Transfusion Blood Bank of the Medical Clinic Tübingen

Bacterial strains

Strain	Source
Escheria coli K12	ATCC
Pseudomonas aeruginosa PA01	ATCC
Staphylococcus aureus HG001	Microbial Genetics, Uni. Tübingen
Staphylococcus aureus Newman	ATCC
Staphylococcus aureus USA300	ATCC

Methods

Bacterial strains, growth conditions and antibiotics

S. aureus strains was grown in Tryptic Soy Broth (TSB, Difco) to the preferred stage as stated in each study. Mueller Hinton Broth (MHB) was used for the MIC determination. IgG from bovine serum, Fetal bovine serum, bovine serum albumin, and fibronectin from bovine plasma were purchased from Sigma. Fetal bovine serum (FBS) and bovine serum albumin (BSA) were dissolved in the Mueller-Hinton Broth (MHB) to attain the preferred concentrations before sterile filtration. Fibronectin and Bovine IgG were resuspended in 0.9% NaCl and sterile filtered before additional dilution in MHB. The PPAPs used in this study, PPAP 23, PPAP 22, and PPAP 53 were all obtained from the laboratory of AG Bernd Plietker. Rom and its derivatives were synthesized by AG Martin Maier.

Peptide synthesis

The amino acid sequences of peptides used in this study are given in Table 1. The peptides were synthesized by Apeptide (Shanghai, China) with a purity of >95%, solubilized in water at 1 mg/ml and stored at −20°C. 20 µM of L15, 30 µM of L13 and 5 µM of geldanamycin were used in *in vitro* studies unless mentioned otherwise.

Antibiotic susceptibility testing

The minimal inhibitory concentration is adopted as the parameter to measure the bacterial susceptibility to the antimicrobial drug (Gemmell *et al.*, 2006). The minimal

inhibitory concentration determined by the microdilution method according to the guidelines of the Clinical and Laboratory Standards Institute document M07-A9 (Rennie *et al.*, 2012). Each antibiotic was serially diluted resulting in 50 µl antibiotic dilution in the respective well of a 96-well microtiter plate, 50 µl bacterial suspension of 10^6 CFU/ml were added to the antibiotic dilution, and growth control. The microtiter plate was incubated at 37°C under continuous shaking for 18 hours or 24 hours before the measurement of OD with a microplate reader (TECAN Infinite M200). The MIC is the lowest concentration of an antibiotic that prevents the visible growth of a microorganism (Wiegand *et al.*, 2008).

Invasion studies in HaCaT and N/TERT-1 cells

The invasion protocol was based on previously established methods (Nguyen et al., 2015, Tribelli et al., 2020). A monolayer of ~106 keratinocytes per well was created by seeding them in a 24 well plate (Greiner, Frickenhausen, Germany). *S. aureus* was grown overnight at 37°C, centrifuged and suspended in invasion medium (DMEM with 10% FBS for HaCaT and K-SFM for NTERT-1 cells). The adherent keratinocytes were washed with PBS and supplemented with invasion medium followed by treatment with 20 µM of L15, 30 µM of L13 or 5 µM geldanamycin (Sigma-Aldrich, Germany) or monoclonal antibodies specific against Hsp90α (α-Hsp90α, Abcam) for 1 h. After 1h, 100 µl of bacterial suspension was added to each well to attain a MOI (multiplicity of infection) of 30 and incubated with cells for 1.5 h. After this, the extracellular bacteria were killed with treatment of 2.5 µg/ml lysostaphin (Sigma-Aldrich, Germany)) for 1.5 h. The cells were then washed, lysed, mechanically detached by scraping, diluted and seeded on TSB plates to quantify the intracellular bacteria.

$CD14^+$ monocyte isolation

Peripheral Blood Mononuclear cells (PBMCs) were isolated by density gradient centrifugation. From the pool of PBMCs, monocytes were isolated by positive selection with anti-human CD14 microbeads (Miltenyi Biotech, Bergisch-Gladbach, Germany) using the protocol from the previous publication (Nguyen et al., 2022). The purity was analysed by flow cytometry on a BD Accuri C6 (BD Biosciences, Heidelberg, Germany)

with anti-human CD14-FITC, CD45-PE and propidium iodide (BD Biosciences, Heidelberg, Germany) and it ranged from 85% to 98%.

Phagocytosis assay

For phagocytosis 10^6 $CD14^+$ monocytes were seeded in 1 ml medium (RPMI supplemented with L-Glutamine and 10% FCS) in a 12 well plate (Greiner, Germany). The cells were pre-treated with peptides/ geldanamycin for 1h at 37 °C in 5% CO2 before the addition of USA300 at MOI of 30 (for 1.5h). The cells were washed once with PBS followed by addition of 20 µg/ml of Lysostaphin for 1.5 h to kill the extracellular bacteria. The cells were scraped, lysed by sonication and serially diluted. The numbers of internalized bacteria were quantified by drop plate method.

Bacterial Growth kinetics

An overnight culture of USA300 was adjusted to OD of 0.01 into a 48 well plate and L15 and L13 or 1X MIC PPAP53 and/or 1% BSA were added to study their effect on growth of bacteria using Varioskan LUX Multimode Microplate Reader. With this instrument, a kinetic measurement of OD578 nm was determined every hour for a total of 24 h, at 37°C with nonstop shaking.

Peptide - Hsp90α interaction studies with immunoblotting

2 µg of each peptide was blotted (dot blot) directly onto the nitrocellulose membrane and blocked with 3% BSA. After being washed, the membrane was then exposed to 20 µg of recombinant Hsp90α protein at a temperature of 4 degrees Celsius for an entire night. α-Hsp90α was used as the primary antibody and alkaline phosphatase conjugated goat-α-mouse IgG (Sigma-Aldrich, Germany) as the secondary antibody. Detection was done using BCIP®/NBT solution (Sigma, Germany).

F-actin formation

5 x 10^4 cells were seeded in black flat bottom 96 well cell culture microplate (Greiner, Germany) and incubated overnight. F-actin was measured using a fluorescently labeled phalloidin, which binds specifically to F-actin, to visualize the F-actin

cytoskeleton. Cells are fixed, permeabilized and then stained with phalloidin conjugated with a fluorescent dye ActinGreen™ 488 ReadyProbes® (Thermo Fischer). The fluorescence intensity of the cells is then quantified using a fluorescence microscope at Excitation/Emission: 495/518 nm (Luqman *et al.*, 2020)

Cytotoxicity studies

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was used to test the cytotoxicity of peptides and geldanamycin to the cells. 5 x 10^5 cells were seeded in a 96 flat bottom well plate and incubated for 2.5h/ 24 h at 37 °C with 5% CO_2. The cells were then incubated with peptides or geldanamycin for 24 h. Next, 10 µl of the MTT labelling reagent is added to each well (final concentration 0.5 mg/ml) and incubated for 4h. Metabolically active cells convert the yellow tetrazolium salt to purple formazan crystals, which were then solubilized using the solubilization solution (DMSO) and quantified using spectroscopy with absorbance at $\lambda_{max}/\lambda_{ref}$ - 570/690 nm (Saising *et al.*, 2018).

Larval studies

The infection experiment was conducted as previously described (Popella *et al.*, 2016). *Galleria mellonella* wax moth larvae, purchased from Feeders & more GmbH, Germany, were sorted based on weight. The studies on larvae were done within a week of purchase. Each group had 10 larvae with weight average of 500mg/larvae. The larvae were injected with bacteria and/or peptides on its last proleg using a BD insulin syringe. The dosage used for the experiment were 60 mg/kg for peptides, 5mg/kg of geldanamycin and 20 mg/kg for vancomycin. Each larva was injected with 10µl of L15 (last left proleg) 1h before administration of 10^6 cfu S. aureus (last right proleg). In case of PPAP studies, the overnight culture of *S. aureus* USA300 was washed with PBS twice to adjust to an OD578 of 0.1, and 10 µl of bacterial suspension, corresponding to a dose of 10^5 CFU was injected to the last left proleg of each larva; and 10 µl of PPAP 23/53, corresponding to a dose of 20 mg/kg was injected to the right proleg of each larva in the treatment group. Untreated larvae and larvae injected with PBS were in the control groups. The larvae were kept at 37 °C and observed every day over the course of 5 days.

Ex vivo killing assay of *Galleria mellonella* larvae

The larvae of *Galleria mellonella* produced about 100 µl of body liquid, which was extracted, filtered, and made sterile. In order to mimic the in-vivo infection assay, the ex vivo killing assay used the same amount of bacterial suspension and PPAP as the infection model. 105 CFU of bacterial inoculum and 10 µg of PPAP were added to 100 µl of larva body liquid to form the treatment group. The controls were untreated larval liquid and larval liquid treated with PBS. The plate was incubated overnight at 37 ºC with shaking at 20 rpm to simulate larva movement. The viability of bacteria in each group was determined by the drop plate method (Herigstad *et al.*, 2001).

Hemolysin activity

The hemolysin activity assay is a method used to determine the ability of bacterial strains to produce hemolysins, which are toxins that lyse erythrocytes (red blood cells). 6mm Whatman empty antibiotic assay discs were saturated with L15 or L13 and left to dry. Once dried, the discs were placed on sheep blood agar plates inoculated with *S. aureus* and incubated at 37°C for 24 hours. Observe the plate for zones of hemolysis, which are clear or yellow areas around the bacterial colonies, indicating the presence of hemolysins. The size and the clearness of the zone of hemolysis will indicate the amount and the activity of the hemolysin produced.

Mouse studies

8 to 10 weeks old female NMRI mice were purchased from Envigo (Venray, Netherlands) and stored under standard light, temperature, and nutrition conditions in the animal facility of the Department of Rheumatology and Inflammation Research, University of Gothenburg. Owing to its low solubility in PBS, L13 was not tested in mice. To study the effect of L15 on *S. aureus* bacteremia, the mice were treated intraperitoneally with L15 (10 mg/kg) in 200 µl of PBS every 12 hours, starting 2-3 hours before inoculation with bacteria (2×10^6 CFU/mouse of *S aureus* Newman) and continuing until the animals were sacrificed. The infection was only done once, and 0.2 ml of *S. aureus* Newman suspension was intravenously injected into the tail vein of the

mice. The mice were regularly weighed every day until they were sacrificed. Upon sacrifice at day 7, kidneys from mice were aseptically removed, homogenized, serially diluted with PBS and spread on agar plates containing 5 % horse blood. Plates were incubated at 37°C for 24 h and bacteria were quantified as CFUs. Before homogenisation, the kidneys were blindly assessed by one investigator (T.J.) for abscesses. A scoring system ranging from 0-3 was used (0- healthy kidneys; 1- 1 to 2 small abscesses on kidneys without structure changes; 2- more than 2 abscesses, but < 75% kidney tissue involved; and 3- large amounts of abscesses with >75% kidney tissue involved) (Ali *et al.*, 2015).

Immune stimulation

PBMCs from 19 healthy donors were isolated and stimulated with the 35µg/ml of L15 and L13 for 20 hours. The secreted cytokines were then quantified using the immunofluorescent bead-based immunoassay LEGENDplex® Human Macrophage/Microglia Panel (13-plex) and the Data Analysis Software from BioLegend. Muramyl Dipeptide (MDP) a NOD-2 ligand was used as the positive control.

Bioinformatics analysis

The 3D structure of Lpl1 was predicted using the protein structure prediction server Robetta using RoseTTAFold modelling method (Baek et al., 2021). The obtained .pdb structure was then visualized in PyMOL by Schrödinger. The homologs of *S. aureus* USA300 Lpl1 in different bacteria were identified using the Protein BLAST tool on the NCBI server. The comparison of different lipoprotein homologs from different bacteria was done in Snap gene using Clustal Omega algorithm.

A well-defined crystal structure of BSA was downloaded from protein data bank (PDB ID: 3V03) (Majorek et al., 2012). The calcium and acetate ions attached to the crystal structure, as well as the water molecules used for crystallization were removed to get a clean structure of the BSA protein. 2D structure of PPAP was prepared and its geometry was optimised by energy minimisation in Chem3D 16.0. The structures are visualized and verified using PyMoL software. The PDBQT files of Protein and ligands were prepared using AutoDock Tools and blind docking was performed in AutoDock

vina (Trott & Olson, 2010). During the preparation of PDBQT files polar hydrogens and Kollman charges are added for both the protein and ligand. The serum albumin was considered as a rigid structure and PPAP as the flexible one during docking.

Statistical analysis

Statistical analysis was performed using GraphPad Prism. Student's t-tests or one-way analysis of variance (ANOVA) were used to determine the statistical significance of the results. Results were considered not significant (ns) if the P value was greater than 0.05. In figures, significant differences are indicated as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; and **** $p < 0.0001$.

Ethical Statement

The studies on human peripheral blood mononuclear cells (PBMCs) from buffy coats was approved by the Ethics Committee of the Medical Association of Westphalia-Lippe and the University of Münster (Approval number 2021-063-f-S) and the Ethics Committee of the Medical Faculty of the University of Tübingen and the Medical Clinic Tübingen (approval number 084/2021BO2). Buffy coats were obtained from the German Red Cross Blood transfusion west (Hagen, Germany) and the Transfusion Blood Bank of the Medical Clinic Tübingen. The Ethics Committee of Animal Research of Gothenburg approved all experiments conducted on mice. The mouse experiments were performed in accordance with the Swedish Board of Agriculture's regulations and recommendations on animal experiments.

Chapter 3

Results

Results

Studies on L15 and L13

L15 reduces USA300 invasion into keratinocytes

It has been shown in our previous papers that lpl cluster be conducive to the invasion of USA300 into HaCaT cells. In the Δlpl mutant the, amount of invasion was 2.5 fold less as compared to the wild type. The invasion protocol was adapted from our previous paper (Tribelli *et al.*, 2020).

Preincubation of L15 and L13 with HaCaT cells reduced the invasion of USA300 into the former by nearly 59.9 and 54.7% respectively. Relative invasion of *S. aureus* into the HaCaT cells reduced from 1 ± 0 (untreated control) to 0.40 ± 0.09 on pre-treatment with L15 and 0.45 ± 0.06 on pre-treatment with L13. Enhanced invasion of USA300 into HaCaT cells were seen on pre-treatment with peptides L11 (1.57 ± 0.20), L26 (1.50 ± 0.17) and L27 (1.55 ± 0.17). Other test peptides didn't affect the invasion activity of bacteria into the cell (Table 1).

Geldanamycin, a benzoquinoid from *Streptomyces hygroscopicus*, was used as a positive control. It has been demonstrated in our previous paper that 5 µM geldanamycin showed significant reduction in USA300 internalisation (Tribelli *et al.*, 2020). Herealso, we could see that geldanamycin showed 75 % reduction of USA300 invasion into the HaCaT cells (relative invasion for geldanamycin group was 0.25 ± 0.13). Pre-treatment of HaCaT cells with α-Hsp90α, also reduced the invasion of *S. aureus* by 89.3% (0.11 ± 0.09) (Fig. 3.1A).

We did the same studies on N/TERT-1 keratinocyte cell line using L15 and L13. N/TERT-1 were a kind gift from Dr. J. Rheinwald, Harvard Medical School, Boston, USA (Dickson *et al.*, 2000). N/TERT-1 cells are frequently employed as an auxiliary for primary keratinocyte cells due to the limited obtainability and high inter-donor inconsistency of the latter. N/TERT-1 cells are immortalised and are shown to work essentially like primary human keratinocytes in expression of host defence genes and proteins, and epidermal differentiation (Smits *et al.*, 2017). Similar pattern was exhibited in N/TERT-1 cells. Intracellular *S.aureus* USA300 relative bacterial load was

significantly reduced on pre-treatment with L15 (0.6 ± 0.05), L13 (0.37 ± 0.10), geldanamycin (0.24 ± 0.04) and α-Hsp90α (0.25 ± 0.04) in N/TERT-1 cells as compared to the untreated control (Fig. 3.1B).

It should be noted that neither peptides (L15/L13) nor geldanamycin showed any inhibitory action towards the cell lines (Fig. 3.2A, B) or *S.aureus* for the duration of the experiment. Also, the addition L15 and L13 didn't influence the growth of *S.aureus* USA300 at 37°C (Fig. 3.2C).

Table 1. Effect of tested Lpl1-derived peptides on their invasion potential, F-actin formation and binding to Hsp90α.

Name	Sequence	No of AA	Relative Invasion	Interaction with Hsp90α	Relative F-actin level
L1	^{34}GKGNETKED	9	1.02 ± 0.34	No	0.95 ± 0.49
L2	^{55}TLDMYPIKNLED	12	1.12 ± 0.40	No	0.99 ± 0.13
L3	^{67}LYDKEGYRDS	10	1.00 ± 0.11	No	1.03 ± 0.1
L4	^{77}EFKKGDKGMWT	11	0.93 ± 0.12	No	0.94 ± 0.08
L5	^{81}GDKGMWTIYTDFAKSNKPGELDDEGMVLNLDRNTR	34	1.59 ± 0.76	No	0.97 ± 0.08
L6	^{85}MWTIYTDFAKS	11	0.98 ± 0.68	No	0.91 ± 0.1
L7	^{88}IYTDFAKSNKPGEL	14	1.10 ± 0.18	No	1.03 ± 0.08
L8	^{96}NKPGEL	6	1.28 ± 0.34	No	0.96 ± 0.05
L9	^{102}DDEGMVLNLD	10	1.0 ± 0.44	No	1.06 ± 0.07
L10	^{113}NTRTAKGHYFVTTFYRNG	18	1.10 ± 0.17	Yes	0.99 ± 0.08
L13	^{119}GHYFVTTFY	9	0.45 ± 0.08	Yes	0.87 ± 0.03
L15	^{116}TAKGHYFVTTFYRNG	15	0.4 ± 0.09	Yes	0.83 ± 0.04
L11	^{116}TAKGHYFVTTFYRNGKLPDEKNYKIEMKNNKIILLDEVKDDKLKQKIENFKFFGQYANLKELRK	64	1.57 ± 0.20	No	1.49 ± 0.07
L12	^{126}FYRNGKLPDEKNYKI	15	0.94 ± 0.11	No	0.98 ± 0.06
L14	^{131}KLPDEKNYKI	10	0.78 ± 0.26	No	0.98 ± 0.08
L16	^{137}NYKIEMKNNKIILLD	15	0.92 ± 0.37	Yes	1.08 ± 0.23
L17	^{141}EMKNNKIIL	9	1.05 ± 0.26	No	1.09 ± 0.20
L18	^{144}NNKIILLDEVKDDKL	15	1.05 ± 0.36	No	1.09 ± 0.22
L19	^{151}DEVKDDKLKQKIENF	15	1.29 ± 0.37	No	0.97 ± 0.11
L20	^{162}IENFKFFGQYAN	12	1.15 ± 0.20	No	1.03 ± 0.11
L21	^{163}ENFKFFGQYANLKELRK	17	1.20 ± 0.25	Yes	1.09 ± 0.13
L22	^{177}LRKYNNGDVSINENVPSYDV	20	1.29 ± 0.13	No	0.97 ± 0.09
L23	^{180}YNNGDVSINENVPSYDVEYKMSNK	24	1.09 ± 0.46	No	0.96 ± 0.05

L24	^{194}YDVEYKMSNK	10	0.95 ± 0.12	No	0.94 ± 0.08
L25	^{204}DEIVKELRSRYNIST	15	1.0 ± 0.06	No	1.03 ± 0.16
L26	^{204}DEIVKELRSRYNISTEKSPILKMHID GDLKGSSVGYRKLEI DFSKRENSKLSVIEFLSYKPAKK	64	1.50 ± 0.17	Yes	1.25 ± 0.13
L27	^{211}RSRYNISTEKSPILKMHIDGDLKGSS VGYRKLEIDFSKRENSK	38	1.55 ± 0.17	Yes	1.36 ± 0.12
L28	^{217}STEKSPILKMHIDGD	15	1.0 ± 0.27	No	0.90 ± 0.04
L29	^{227}HIDGDLKGSSVGYRK	15	1.21 ± 0.33	No	0.90 ± 0.05
L30	^{246}FSKRENSKLSVIEFL	15	1.24 ± 0.32	No	0.96 ± 0.1
	Control: Geldanamycin		0.25 ± 0.13	NA	0.88 ± 0.03

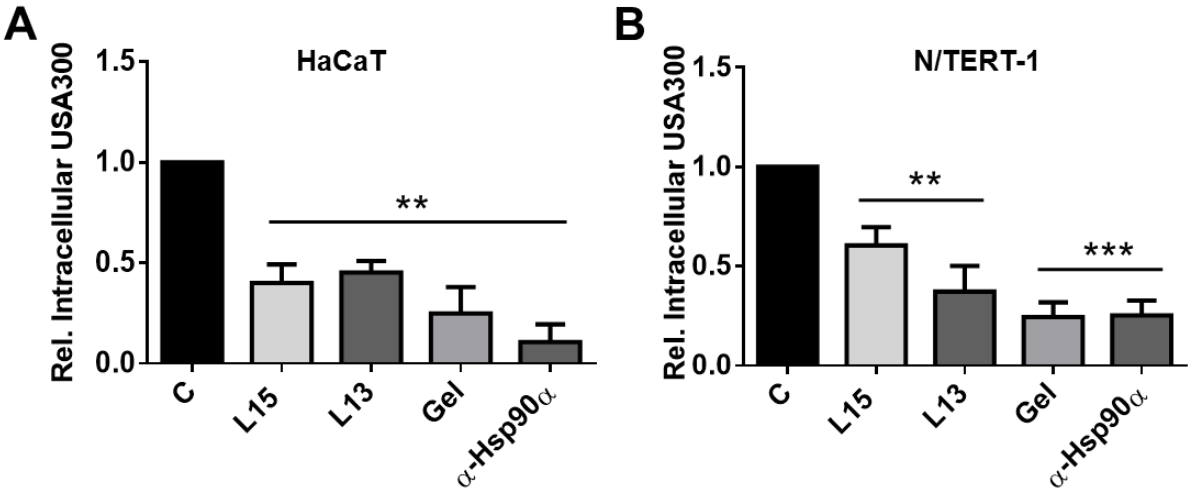


Figure 3.1. L15 and L13 inhibits the invasion of *S.aureus* USA300 into (A) HaCaT and (B) N/TERT-1 cell lines upon pretreatment for 1.5h. Pretreatment of cells with geldanamycin, or with α-Hsp90α (Hsp90α) antibody also inhibited USA300 invasion into the keratinocytes. Error bars show standard deviation of the mean of 3 biological replicates. P values were obtained by student's T-test. : * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; and **** $p < 0.0001$.

L15 and L13 directly interacts with Hsp90α

Lpl1 was shown to directly interact with Hsp90α in our previous studies (Tribelli *et al.*, 2020). Here also, we did the same experiment with the peptides. The synthetic peptides were directly blotted on the nitrocellulose membrane and checked for its binding to Hsp90α using alkaline phosphatase assay. We could see direct interaction of L15/L13 and purified Hsp90α using dot blot studies (Table 1). Other peptides which showed direct interaction with Hsp90α were L10, L16, L21, L26 and L27. No interaction was observed with Bovine serum albumin (BSA) which was used as a control.

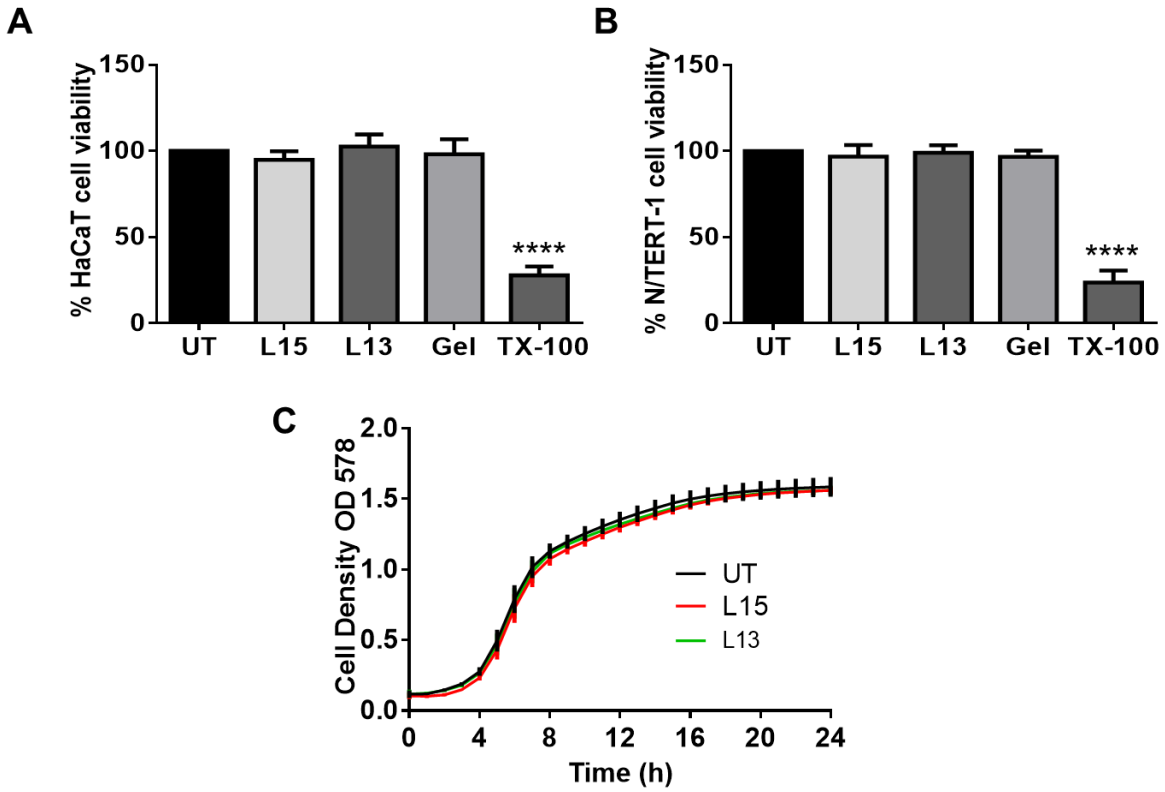


Figure 3.2. L15, L13 and geldanamycin doesn't have any effect on cell lines (A) HaCaT and (B) N/TERT-1 during treatment time. (C) L13, L15 doesn't affect the USa300 growth in TSB at 37 ℃.

L15- Hsp90α interaction reduces F-actin levels in keratinocytes

The mother protein lpl1 was shown to boost the F-actin levels in HaCaT cells in our previous paper (Tribelli *et al.*, 2020). It is also reported that 17-allylamino-demethoxy-geldanamycin (17-AAG) decreased the F-actin levels upon Hsp90 inhibition in breast cancer cells (Taiyab & Rao Ch, 2011). We checked if L15 is affecting the actin polymerisation, since it is interacting with Hsp90 α. Indeed, a significant reduction in F-actin levels were seen in both keratinocytes on treatment with L15, L13 and geldanamycin (Fig. 3.3A, B). In HaCaT cells, relative F-actin levels went down to 0.83 ± 0.04 for L15, 0.87 ± 0.03 for L13 and 0.88 ± 0.03 for geldanamycin. Relative F-actin levels in N/TERT-1 cells for L15, L13 and geldanamycin were 0.9 ± 0.02, 0.81 ± 0.03 and 0.82 ± 0.03 respectively. As expected enhanced F-actin levels were seen when L11, L26 and L27 were introduced to HaCaT cells (Table 1).

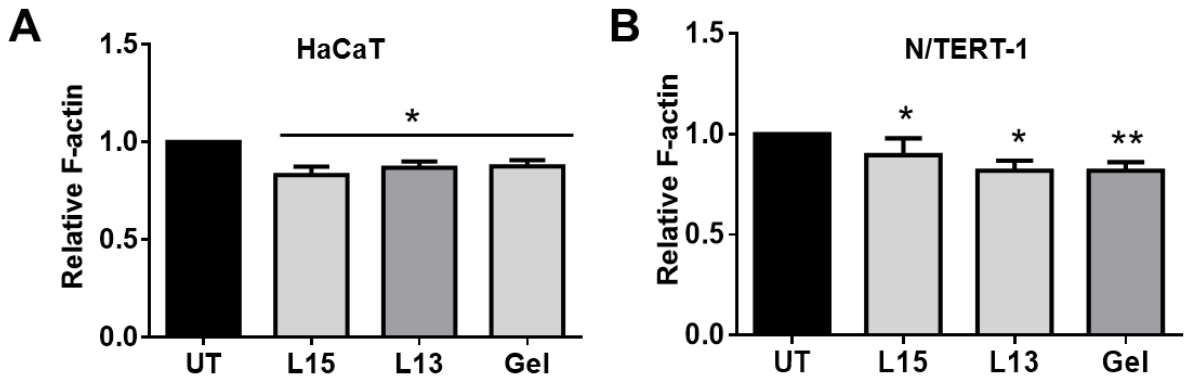


Figure 3.3. L15, L13 and geldanamycin interaction with Hsp90α effects the F-actin formation in (A) HaCaT and (B) N/TERT-1 cells. Error bars show standard deviation of the mean of 3 biological replicates. P values were obtained by student's T-test. : * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; and **** $p < 0.0001$

L15 and L13 are non-toxic to cells *in vitro*

10-50 µM of L15 and L13 were checked for its cytotoxicity on HaCaT- human Keratinocyte cell line, HEK-human embryonic kidney cells, HT-29 - human colorectal adenocarcinoma cell line and MM6 cell line - human monocytic cell line for a 24h time period using MTT assay (Fig. 3.4A-D).

The percentage of viable cells on treatment with L15 for 24h ranged from 90.36 ± 2.89 (10µM) to 93.56 ± 3.96 (50µM) for HaCaT cells and 86.53 ± 6.23 (10µM) and 93.12 ± 4.96 (50µM) for HEK cells. For HT-29 cells, L15 showed cell viability from 100.32 ± 4.07 % and 97.03 ± 4.44 % for 50 and 10 µM respectively. MMT studies on MM6 cells indicated % viability of 111.45 ± 7.96 for 50 µM and 101.31 ± 3.52 for 10µM L15.

Treatment with 50 µM of L13 resulted in percentage cell viability of 92.62 ± 6.08 for HaCaT, 89.0 ± 5.52 for HEK, 97.07 ± 5.55 for HT-29 and 90.01 ± 8.58 for MM6 cells. 10 µM of L13 showed a cell viability of 98.03 ± 8.62 % (HaCaT), 91.47 ± 8.21 % (HEK), and 93.36 ± 4.95 % (HT-29) and 104.65 ± 10.48 % (MM6).

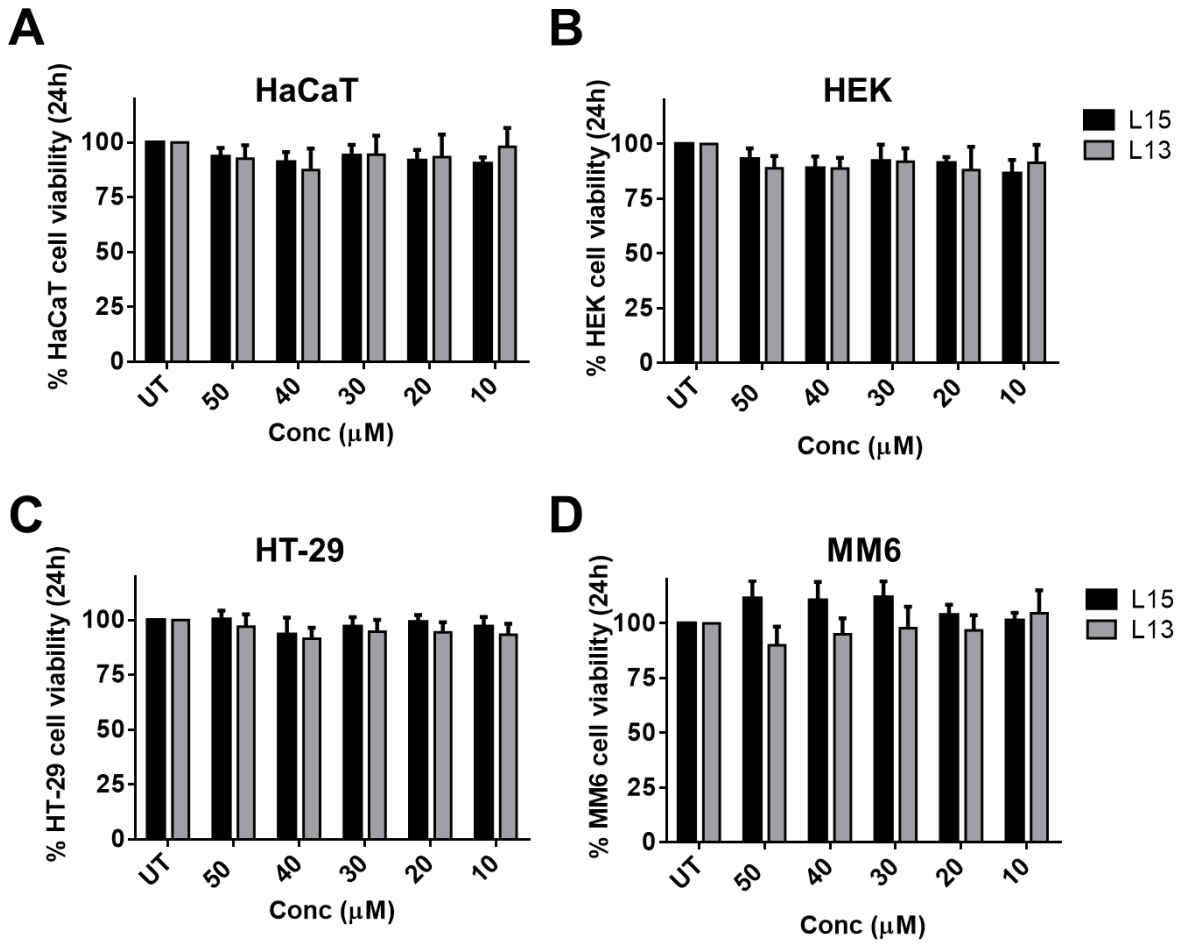


Figure 3.4. L13/L15 is non-toxic to (A) HaCaT, (B) HEK, (C) HT-29 and (D) MM6 cells *in vitro*. Error bars show standard deviation of the mean of 3 biological replicates. P values were obtained by student's T-test. : * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; and **** $p < 0.0001$.

L15/L13 rescues larvae from *S. aureus* infection

The larvae of Galleria *mellonella* or greater wax moth has been well recognized as an experimental model to study the virulence of different pathogens as well as to evaluate the efficacy of various anti-microbial compounds. The large size of the larvae warrants easy handling and direct injection of inoculate into the larval hemocoel. The dosage used for the experiment were 60 mg/kg for peptides, 5mg/kg of geldanamycin and 20mg/kg for vancomycin. Each larvae were injected with 10µl of L15 (last left proleg) 1h before administration of 1-5 $x10^6$ cfu *S. aureus* (last right proleg).The larvae were maintained at 37 °C and observed for its mortality every day for the study period of 5 days. For *E.coli* K12 and *P. aeruginosa* PA01, the MOI used was 10^6 cfu/larvae and 10^2 cfu/larvae respectively (Zheng *et al.*, 2017, Brochado *et al.*, 2018). The larvae were injected with 10µl of L15/L13/geldanamycin 1 h before inoculating *S.aureus* USA300/

Newman/ HG001 into its hemocoel. The larvae were then checked for its mortality for 5 days.

Larvae were injected with different concentrations of (Fig. 3.5A) L15 and (Fig. 3.5B) L13 to find the optimum dosage against USA300 infection. From the results, the optimum dosage was decided as 60mg/kg. Fig. 3.5C shows the larvae infected by *S. aureus* USA300. All infected untreated larvae died by the end of 3rd day. On day 5, L15 could rescue 30% and L13 could rescue 23.33 % of the larvae. Geldanamycin was unsuccessful in saving larvae from infection (they all died by 3rd day). Vancomycin rescued 100% of the larvae and was used as a positive control.

Similar results were obtained for *S. aureus* HG001 infection (data not shown). The percentage larvae alive on 5th day for L15 and L13 were 33.33 and 26.67 % respectively. All infected untreated larvae died by the end of 3rd day. L15 and L13 showed higher rescuing activity for larvae when infected with *S. aureus* Newman (Fig. 3.5D). L15 was able to save 36.67 % of larvae whereas L13 saved 46.67%. The tested doses of peptides, geldanamycin and vancomycin were non-toxic to larvae.

We also tested whether L15 or L13 could affect the pathogenicity of *S. aureus*. A simple disc diffusion assay was done, where *S. aureus* added to pre-impregnated L15 or L13 discs were incubated on sheep blood agar plate overnight at 37^{o}C. The pathogenicity was correlated with the haemolytic activity of the bacteria and the halo formed on the plate was measured and compared with the control. The result suggested that the expression of α-haemolysin or the pathogenicity remained unaffected in *S. aureus* on treatment with L15 and L13 (Fig. 3.6).

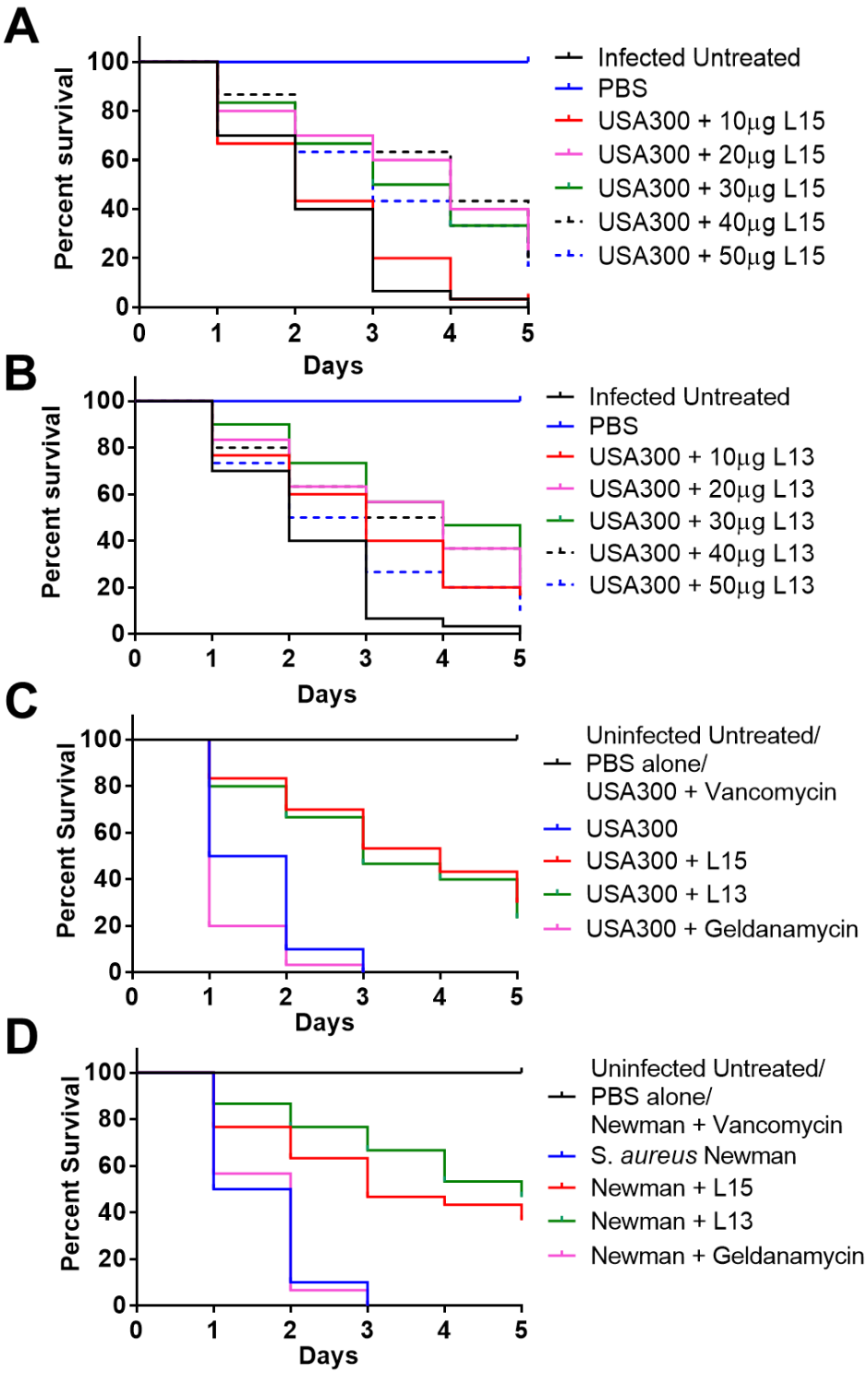


Figure 3.5. L15/L13 rescues larvae from *S.aureus* infection. Each group consists of 10 larvae with weight average of 500mg/larvae, and were administered with bacteria and/or peptides on its last proleg using a BD insulin syringe. Larvae were injected with different concentrations of (A) L15 and (B) L13 to find the optimum dosage against USA300 infection. Each larvae were injected with 10μl of L15 (last left proleg) 1h before injection of 10^6 cfu *S. aureus* (last right proleg) (C) USA300 and (D) Newman. The larvae were maintained at 37 °C and checked for its mortality every day for the study period of 5 days. Total of 3 biological replicates are represented in the graph.

Mice studies

To study the effect of L15 treatment on *S. aureus* systemic infection, NMRI mice infected with *S. aureus* Newman were treated with L15 (10 mg/kg) and clinical outcomes were monitored during the course of disease. Mice infected with *S. aureus* started to lose weight after infection. The mice treated with L15 had significant better weight development during the whole course of infection compared with the control mice who received PBS or distilled water (Fig. 3.7A). No significant difference was found regarding the bacterial load in kidneys (Fig. 3.7B). Regarding the mortality rate, forty percent of animals in the control group died, whereas all mice treated with L15 survived during 7 days of observation time (Fig. 3.7C), suggesting that L15 treatment prevents the lethal *S. aureus* bacteremia.

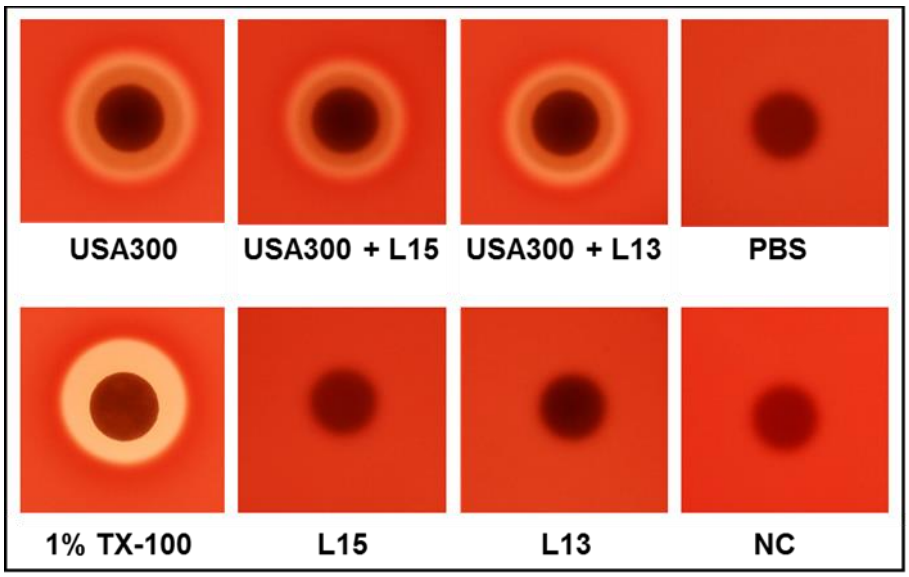


Figure 3.6. L13, L15 doesn't affect the hemolysin activity of USA300

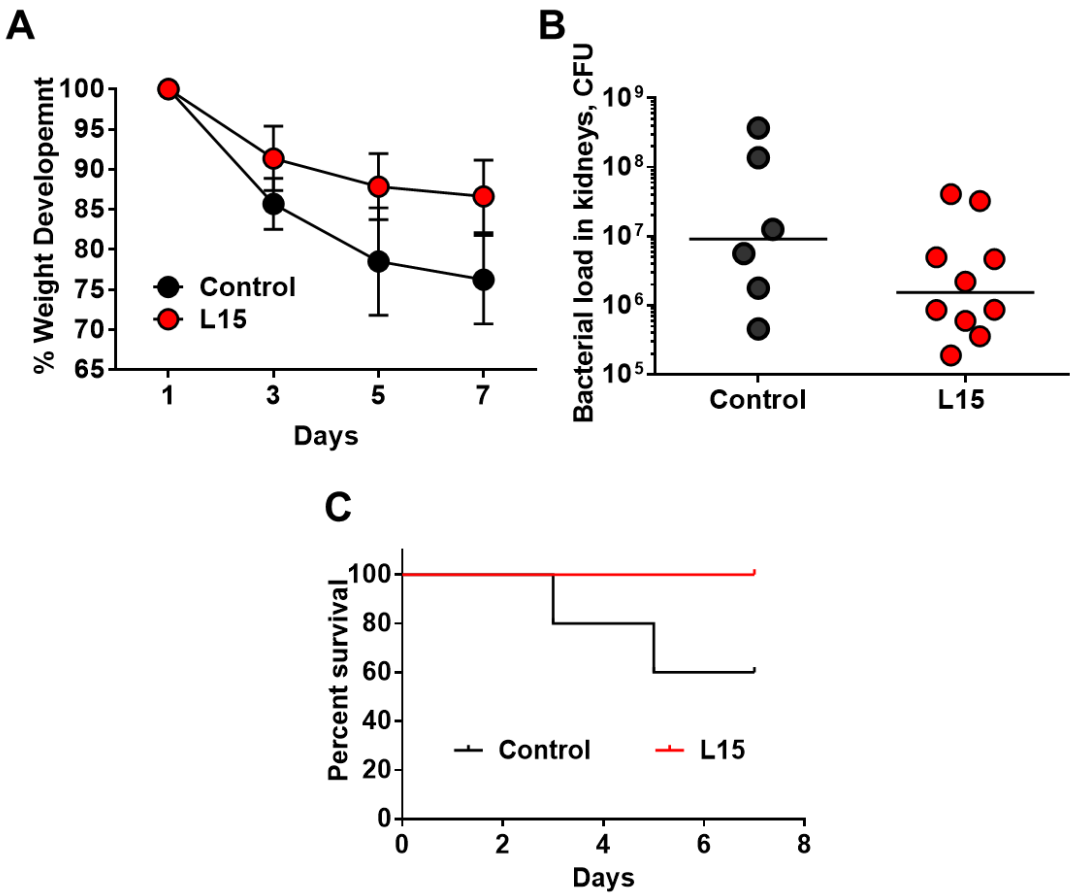


Figure 3.7. L15 treatment reduces systemic *S. aureus* infection. NMRI mice infected intravenously with *S. aureus* Newman strain ($2x10^6$ CFU/mouse) were treated with L15 intraperitoneally (10mg/kg) or PBS for control starting two hours before inoculation and continuing twice daily until animals were euthanized on day 7. (A) Weight development observed during 7 days. (B) Bacterial load in kidneys on day 7 post-infection. (C) Survival graph of mice infected with *S. aureus*.

Multiple sequence alignment

The homologs of *S.aureus* USA300 Lpl1 in different bacteria were identified using the Protein BLAST from NCBI database. The comparison of different Lpl (Lpl1-9) in *S. aureus* and the lipoprotein homologs from different bacteria were done in Snap gene using Clustal Omega algorithm.

The homolog identification of Lpl1 in USA300 using Protein BLAST revealed lipoproteins in different bacteria with high similarity (Fig. 3.8). Lipoproteins from *Staphylococcus hyicus, Staphylococcus schweitzeri* and *Staphylococcus argenteus* showed a percentage identity of 88.64, 97.75 and 88.72 respectively. The corresponding L15 and L13 homolog sequences in these lipoproteins were exactly

similar. *Listeria monocytogenes* had a lipoprotein with sequence similarity of 80.49% with that of Lpl1, whereas the same was 74.62 % for *Lactobacillus ruminis*. *Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* also exhibited highly similar lipoprotein with percentage sequence similarity of 75.76, 90.51 and 85.05 respectively. The corresponding L15 homolog of *Pseudomonas aeruginosa* was exactly similar to L15 of *S. aureus*.

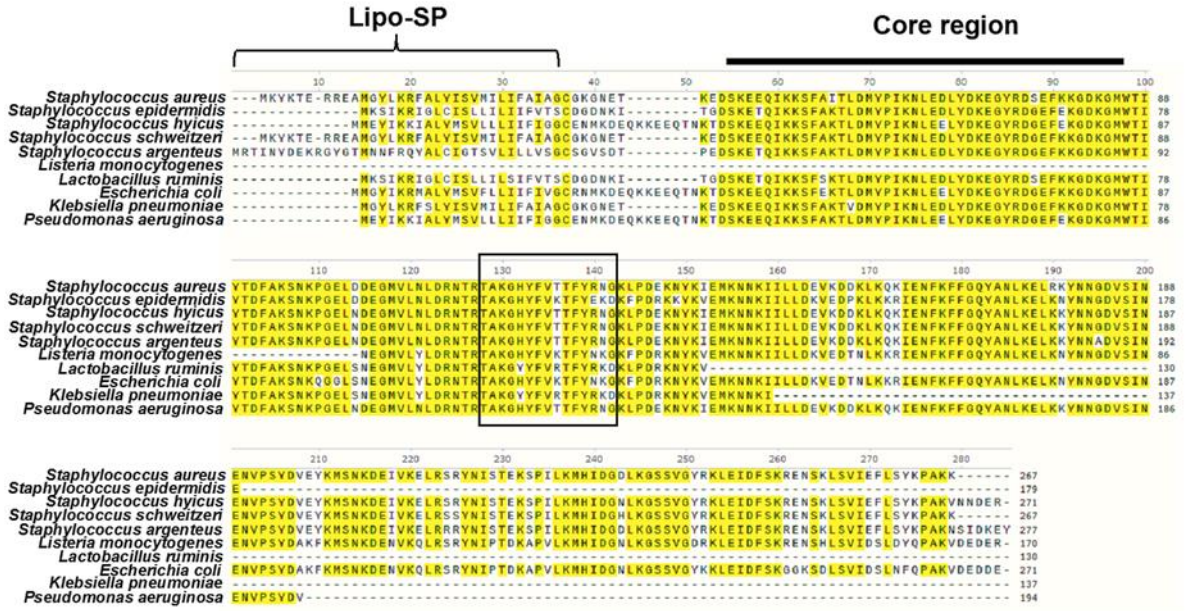


Figure 3.8. Multiple sequence alignments of Lpl1 from *S. aureus* USA300 with other bacteria. These include *Staphylococcus epidermidis* SE62, *Staphylococcus hyicus* NCTC 8294, *Staphylococcus schweitzeri* NCTC 13712, *Staphylococcus argenteus* B3-25B, *Listeria monocytogenes* ATCC 15313, *Ligilactobacillus ruminis* ATCC 27780, *Escherichia coli* NCTC 9001, *Klebsiella pneumoniae* NCTC 9633 and *Pseudomonas aeruginosa* PA216. The lipoprotein signal peptide is indicated by the bracket, the conserved core region by the bar and the L15 sequence is boxed.

All the 9 Lpls in USA300 shared highly similar core region, but the corresponding L15 sequences were not so similar (Fig. 3.9). Lpl2, Lpl4 and Lpl7 showed more similarity to Lpl1 with a percentage identity of 76%.

It should also be noted that the trypsin digestion of Lpl1 (by ExPASy PeptideCutter tool) does not yield L13 or L15 (Gasteiger *et al.*, 2005). The peptides to our knowledge, do not inhibit the growth of bacteria, do not selectively affect any metabolic pathways in the latter and therefore might have a lower chance of resistance development.

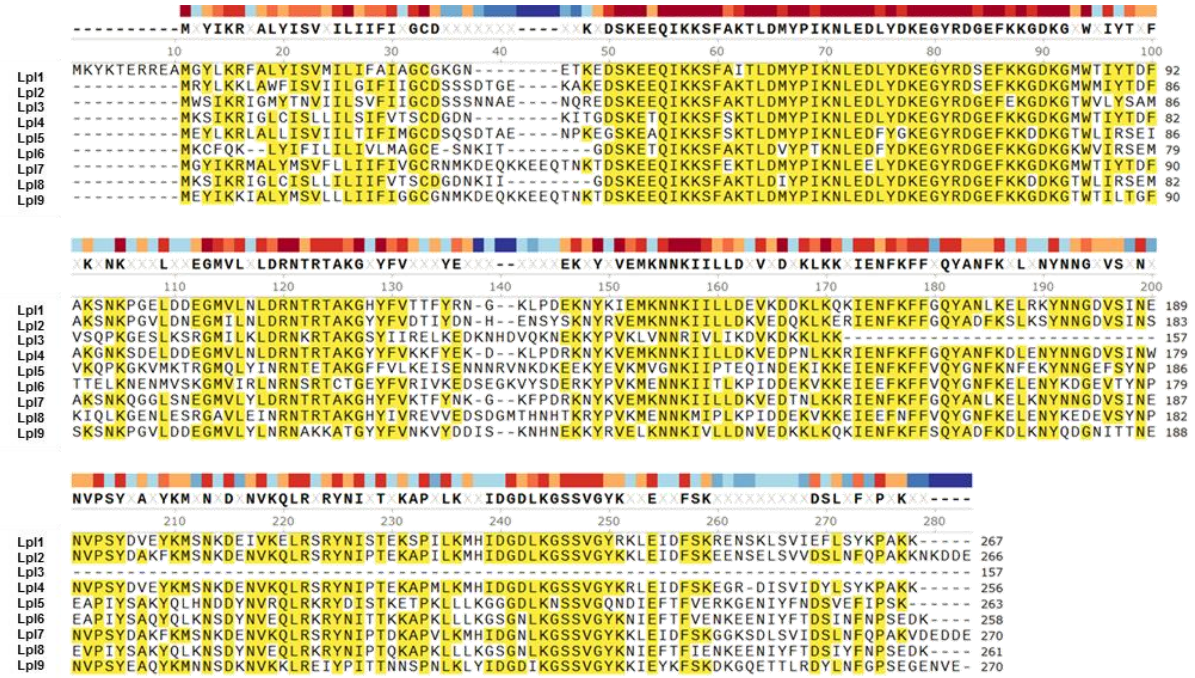


Figure 3.9. Multiple sequence alignments of Lpl1 – Lpl9.

L15/L13 couldn't rescue larvae from *E.coli* and *P. aeruginosa* infection

The sequence similarity of Lipoproteins in *E.coli* and *P. aeruginosa* prompted us to check whether L15 had any protective effect in infections caused by these pathogens. Unfortunately, the peptides failed to rescue the larvae from infective death.

Each larvae were infected with, 10^6 cfu of *E.coli* K12 after pre-treatment with 10µl of the peptides. L15 and L13 were not able to rescue the larvae from *E.coli* K12 infection (Fig. 10A). *E.coli* infection alone killed all the larvae by 5th day, whereas the larvae treated with L15 and L13 died by 4th and 3rd day respectively.

Similar protocol was followed for *P. aeruginosa* PA01 infection.10^2 cfu of *P. aeruginosa* PA01 was injected into the larvae coelom preceded by L15/L13 injection. PA01 infected larvae died by 4th day whereas those pre-treated with L15 and L13 died on the 3rd day. L15 and L13 were unsuccessful in rescuing of Larvae from *P. aeruginosa* infection (Fig. 3.10B). This suggests that the peptides L13 and L15 have a narrow spectrum of activity and it can be used specifically against *S.aureus* strains.

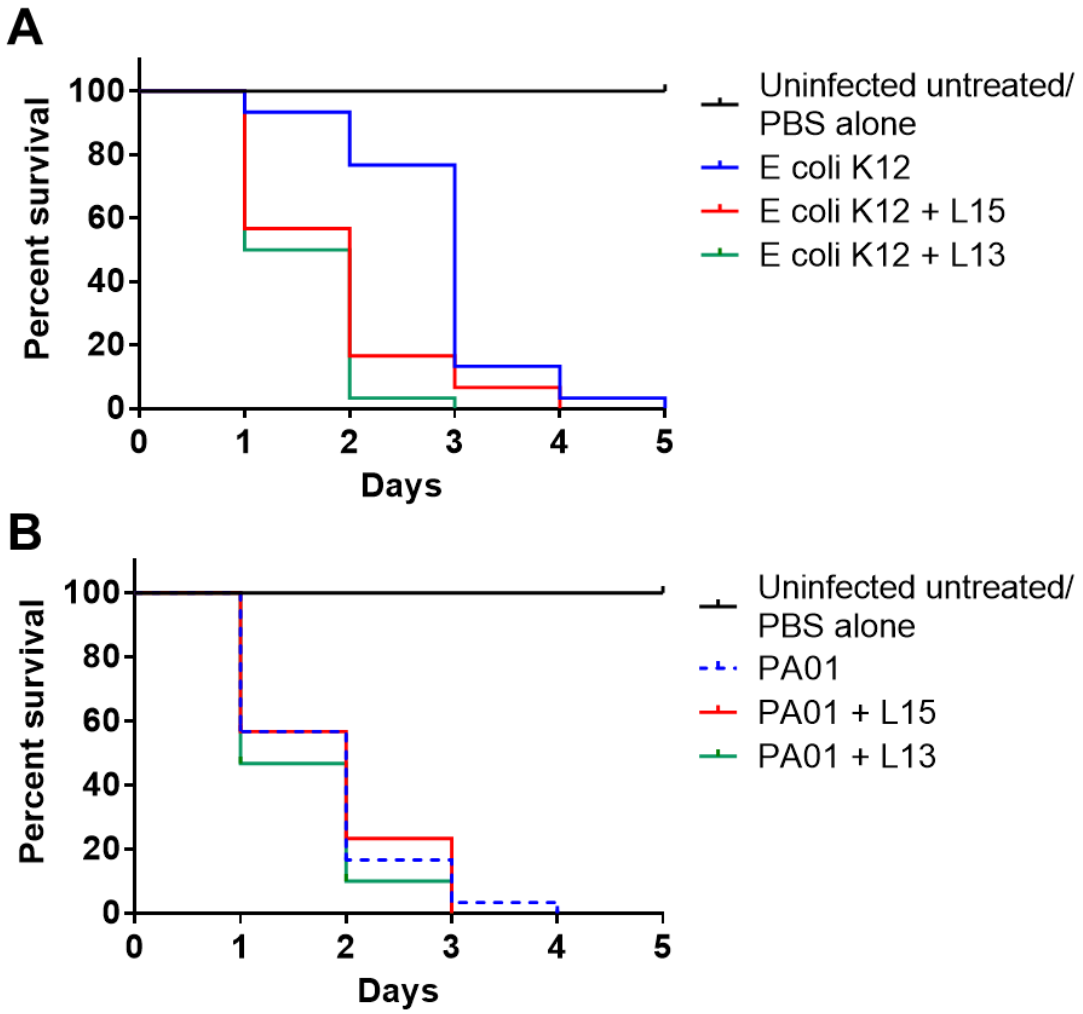


Figure 3.10. L15/L13 couldn't rescues larvae from (A) *E. coli* K12 and (B) *P. aeruginosa* PA01 infection. Ten wax moth larvae per group with weight average of 500mg/larvae were injected with bacteria and/or peptides on its last proleg using a BD insulin syringe. Each larvae were injected with 10µl of L15 (last left proleg) 1h before administration of bacteria and checked for its rescual efficacy for 5 days. Total of 3 biological replicates are represented in the graph.

L15 didn't cause any immune stimulation in human PBMCs

Since L15 showed good results in mice model, we tested the immune stimulation activity of L15 in human PBMCs. Here, the buffy coat blood samples from healthy individuals (attained from Transfusion Blood Bank of the Medical Clinic Tübingen) were stimulated with L15 peptides to induce IL-6, IL-12p790, IL-23, and TNFα, IL-1β, IL-1RA, and IP-12 for 20h. The secreted cytokines were then measured using the immunofluorescent bead-based immunoassay LEGENDplex® Human Macrophage/Microglia Panel (13-plex) and Data Analysis Software from BioLegend. L15 was relatively inert with respect to cytokine inducing activity in PBMCs (Figure

3.11). Muramyl Dipeptide (MDP) which stimulates the NOD-2 receptors was employed as the positive control.

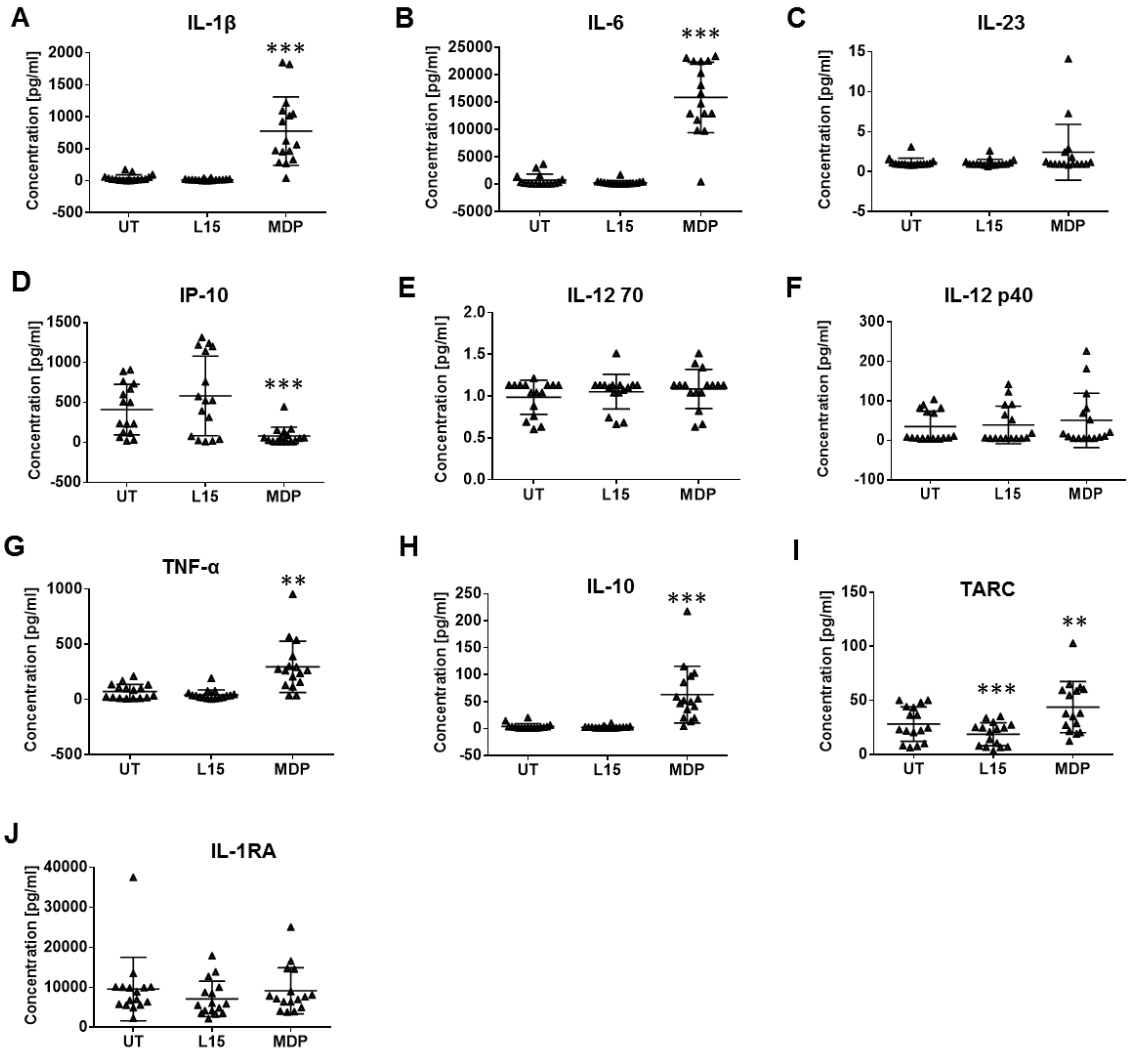


Figure 3.11. Cytokine quantification of PBMCs stimulated by L15, showed that the L15 didn't cause any immune stimulation at the tested concentration. Statistic significances were calculated between the peptide treated cells compared to control by using one-way ANOVA analysis with Tukey's multiple comparison test: *p< 0.05, **p<0.01, ***p<0.001, ns > 0.05.

Pretreatment with L15 and L13 reduced the phagocytosis of USA300 in CD14^{+} monocytes

Similarly like the keratinocytes, L15 and L13 could also reduce the phagocytosis of USA300 into monocytic cells (Figure 3.12A). The relative phagocytosed USA300 count in monocytic cells were 0.77 ± 0.08 for L15, 0.38 ± 0.07 for L13 and 0.86 ± 0.17 for

geldanamycin as compared to the untreated control (1.00 ± 0.0). Geldanamycin didn't significantly affect the phagocytosis of USA300 into the host cells (p value = 0.2).

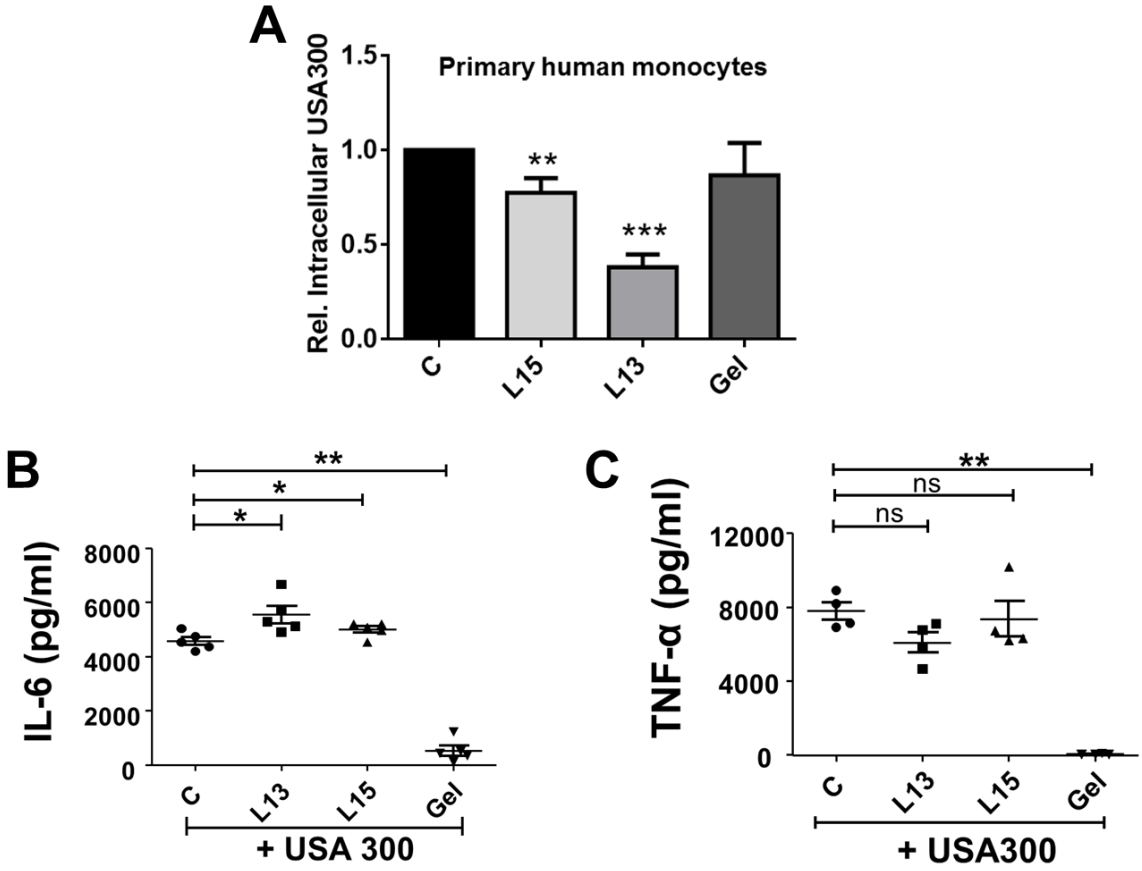


Figure 3.12. (A) Effect of L15 and L13 on S. aureus USA300 phagocytosis by primary human CD14+ monocytes. Effect of L13 and L15 on host innate immune response of 4 donors were determined. Release of (B) IL-6 and (C) TNF-α in the supernatant of *S. aureus*-infected PBMCs was quantified 20 h after stimulation with L13 or L15 or geldanamycin. C indicates control cells without peptide pretreatment. Statistic significances were calculated between the peptide treated cells compared to control by using one-way ANOVA analysis with Tukey's multiple comparison test: *p< 0.05, **p<0.01, ***p<0.001, ns > 0.05.

The peptides effect the IL-6 immune response of PBMCs to *S.aureus* but not TNF-α response

We stimulated the PBMCs of 5 healthy donors with USA300 in combination with/without peptides. Both the peptides were able to significantly bring up the IL-6 cytokine production in PBMCs, but not TNFα production. Geldanamycin inhibited both IL-6 and TNFα release in infected PBMCs (Figure 3.12B, C).

L15 and L13 in combination with different TLR stimulants couldn't rescue larvae from USA300 infection

L15 and L13 was injected in combination with different TLR ligands in *S. aureus* infected larvae to see if their protective effect can be enhanced with the ligands. The ligands used were Muramyl di-peptide or MDP – ligand for TLR2/TLR4, Class A CpG oligonucleotide or CpG ligand for TLR9 and ssRNA for TLR7. Unfortunately, the combination treatment could not enhance the protective effect of L5 and L13 (Figure 3.13).

How the peptides rescue the larvae and mice from infection is not completely clear. It may be the case that the peptides are competing with the Lpl proteins on the surface of *S. aureus* for binding to the Hsp90 receptor. This could cause an indirect neutralization of the Lpl proteins, and in turn reduced pathogenicity. This is in consistence with our earlier data showing that deletion of the lpl genes markedly reduced the pathogenicity of *S. aureus* in a mouse kidney abscess model (Nguyen et al., 2015).

In summary, we have identified small peptides L15 and L13 that have huge potential against *S. aureus* infection which we have proved using *in vivo* models of *Galleria mellonella* and mice. *In vitro* studies indicated the peptides reduced invasion of *S. aureus* into keratinocytes by reducing the F-actin levels and possibly thereby reducing the endocytosis of the said bacteria. Future works will focus on understanding the mechanism of how these peptides protect the host from *S. aureus* infection.

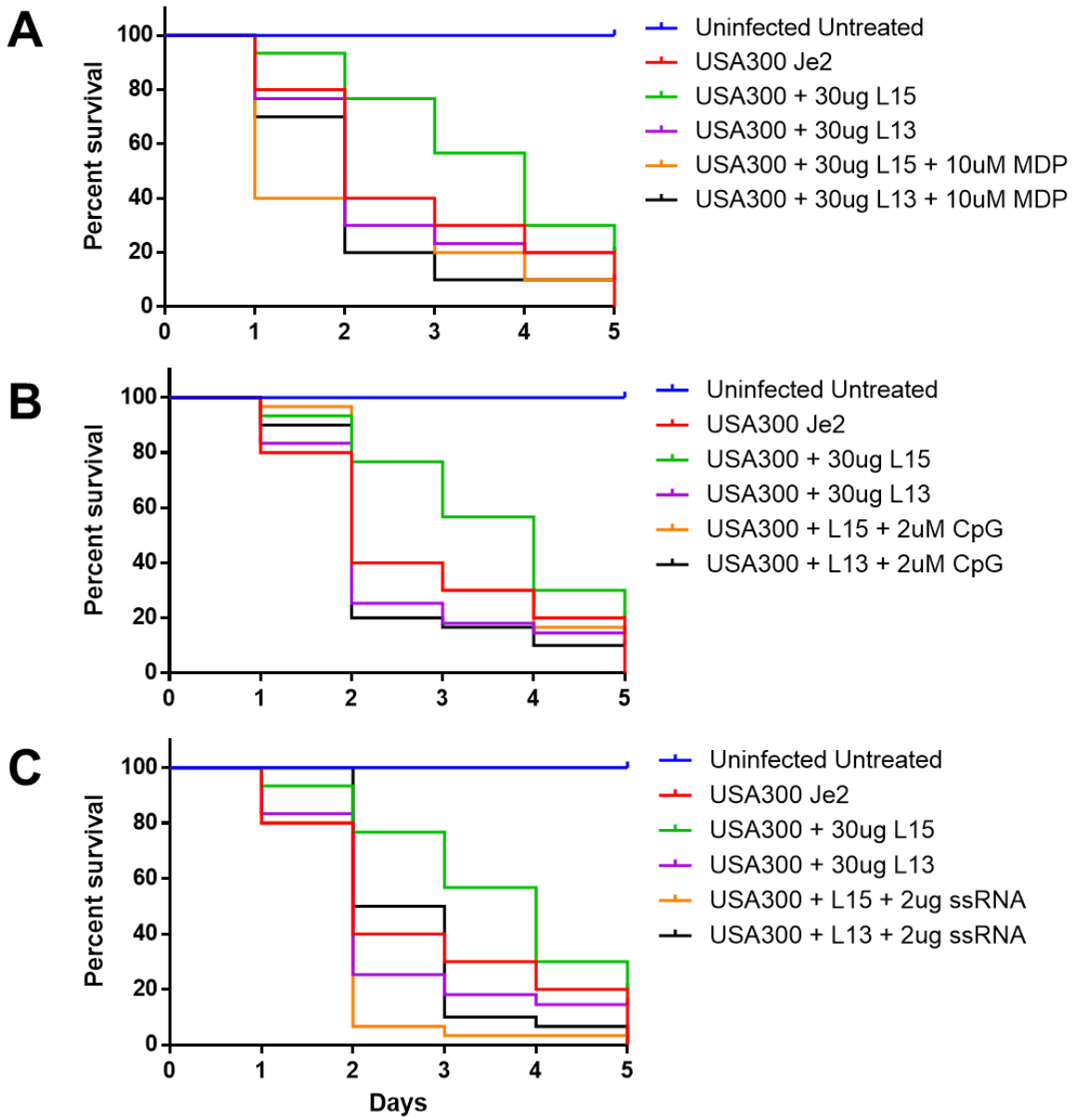


Figure 3.13. L15/L13 in combination with different TLR ligands (A) MDP, (B) CpG and (C) ssRNA couldn't rescues larvae from *S. aureus* infection. Ten wax moth larvae per group with weight average of 500mg/larvae were injected with bacteria and/or peptides on its last proleg using a BD insulin syringe. Each larva was injected with 10µl of L15 (last left proleg) 1h before injection of bacteria. The larvae were maintained at 37 °C and observed for its mortality every day for the study period of 5 days. Total of 3 biological replicates are represented in the graph.

Studies on Rhodomyrtone

Potassium derivative of Rom, FH-54, is active against Rom resistant *S.aureus*

The MIC values of *S.aureus* HG001 and Rom^R strain against different Rom derivatives are listed in Table 2. The IC50 values of the most promising Rom derivatives in HL60 cell lines were also determined by MTT assay. A derivative of Rom, called FH-54 was found to be active against even the Rom^R strain with MIC of 1 µg/mL. FH-54 was also found to be less cytotoxic to HL60 cell lines as compared to Rom.

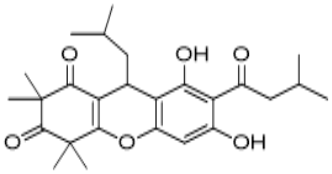
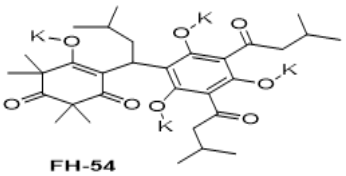
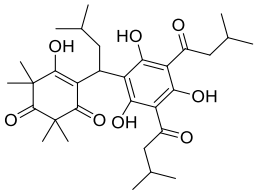
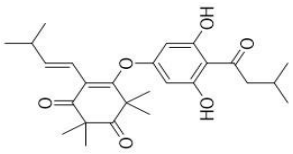
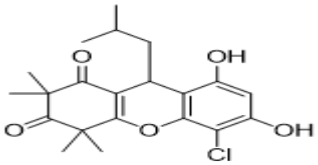
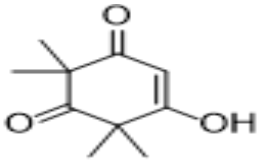
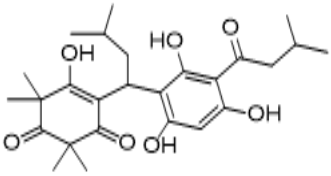
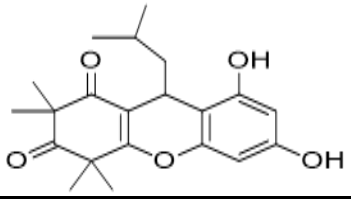
Rom resistance is facilitated by contact of PG with Rom, which nullifies its antibacterial activity

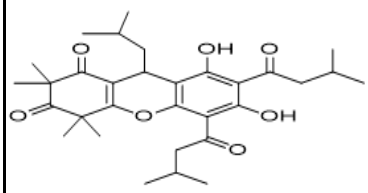
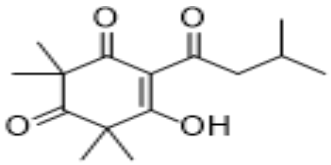
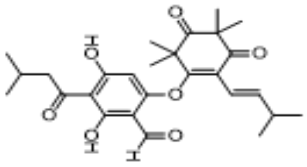
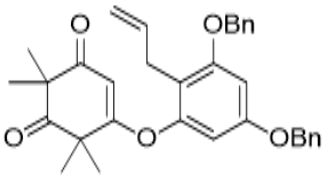
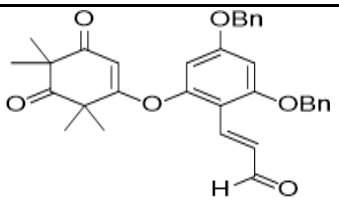
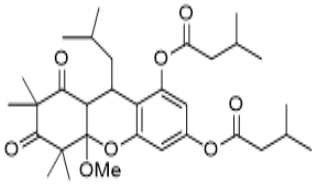
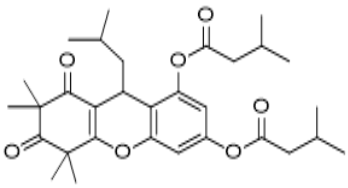
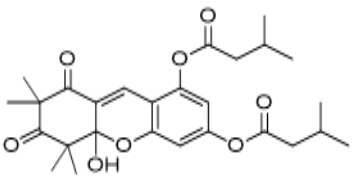
The deletion of *farE* in both wt HG001and Rom^R made them supersensitive to Rom. The MIC of Rom in HG001 reduced to half (1 to 0.5 µg/ml) upon farE deletion, whereas the MIC went from >128 to 0.5 µg/ml in Rom^R strain after the same deletion. This gives us proof that farE is important for resistance in Rom^R strains.

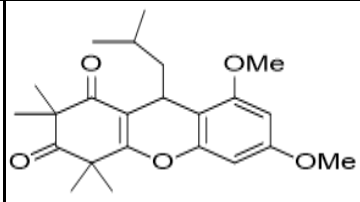
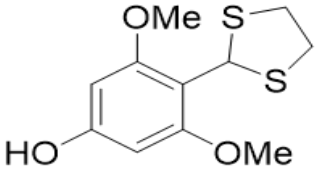
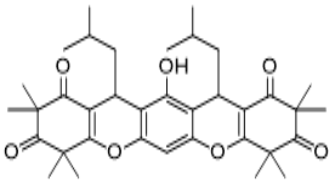
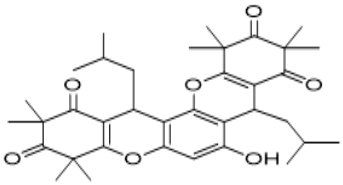
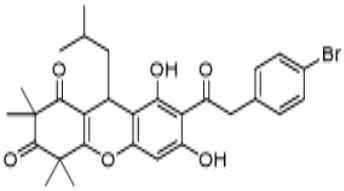
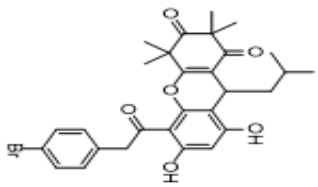
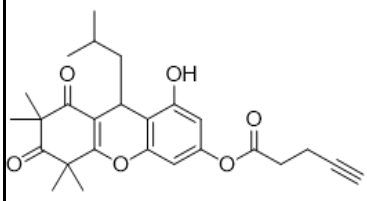
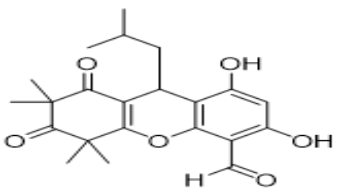
Rom's antimicrobial activity could be neutralized by 2X concentrated Rom^R supernatant and 20X concentrated HG001 supernatant. This advocates that the elements causing Rom resistance are the present in both the parental strain and the Rom^R mutant but in different concentrations. This prompted us to conduct a lipidomic analysis on the supernatant and pellet wash of HG001 and Rom^R strain. The Rom^R strain released more PG and LysI-PG as compared to HG001. The total amount of PG in supernatant of HG001 was 268 ng/OD and in Rom^R was 2244 ng/OD. Rom^R released more than 7 times LysI-PG in comparison to HG001 (HG001: 268 ng/OD and RomR: 2244 ng/OD).

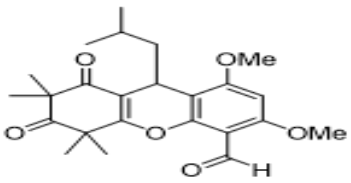
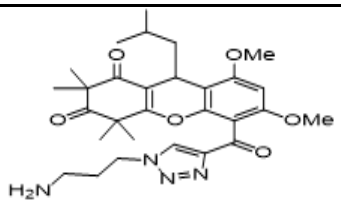
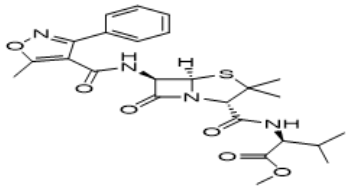
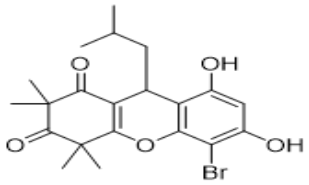
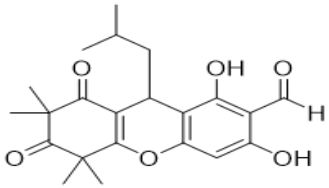
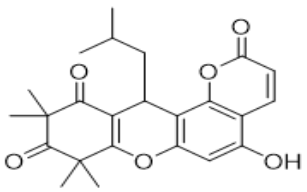
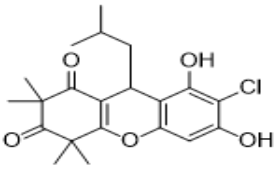
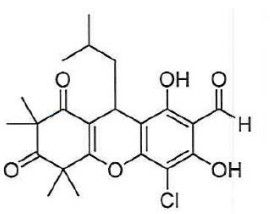
The deletion of *mprF* in Rom^R strain, didn't change its resistance towards Rom, disproving the hypothesis that *mprF* had any effect on Rom resistance. SLS/DLS experiment showed that Rom interacts with PG vesicles leading to an increase in the vesicle diameter (diameter changed from100 nm to 200 nm). ITC studies revealed that the interaction of Rom and PG happens really fast with an enthalpy change (ΔH) of 6.10 kcal/mol, equilibrium binding constant of 2.30 ±0.747 and a favorable (negative) entropy change. Moreover, resistance towards rom didn't cause any cross-resistance to known antibiotics.

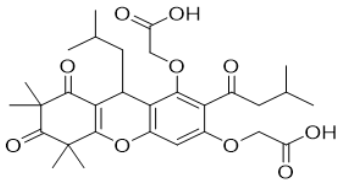
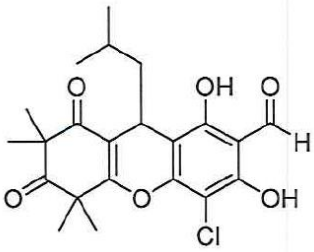
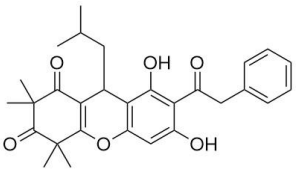
Table 2. MIC values of Rom and its derivatives towards *S.aureus* HG001 and its Rom^R strain.

Compounds	Structure	MIC (µg/mL)	
		HG001	Rom^R
Rom HL60 IC50 (mg/ml) = 5		1	>64
FH-54 HL60 IC50 (mg/ml)= 44	 FH-54	1	1
2-152 HL60 IC50 (mg/ml)= 31		0.5	8
2-171 HL60 IC50 (mg/ml) = 4		0.5	1
MU_118		4	2-4
2-26 (Syncarpic acid)		> 32	> 32
2-27		16	64
2-151		16	16

Compounds	Structure	MIC (μg/mL)	
		HG001	Rom^R
2-153		32	32
2-162 (Leptospemone)		>32	>32
2-175		>32	>32
2-181		>32	>32
2-182		>32	>32
2-185		>32	>32
2-187		>32	>32
2-189		>32	>32

Compounds	Structure	MIC (µg/mL)	
		HG001	Rom^R
2-212		>32	>32
SR_01		>64	>64
MU_08_N1		64	>64
MU_08_N2		>64	>64
MU_15		2	>64
MU_19		16	>64
MU_20		32	32
MU_25		32	32

Compounds	Structure	MIC (µg/mL)	
		HG001	Rom^R
MU_35		>64	>64
MU_77		>64	>64
MU_81		>64	>64
MU_96		8	>64
MU_98		8	>64
MU_99		>64	>64
MU_118 NP		16-32	16
MU_124		>64	>64

Compounds	Structure	MIC (µg/mL)	
		HG001	Rom^R
MU_126		>64	>64
MU_124		>64	>64
MU_141		1	4

Rom is active against pathogenic Gram-positive gut anaerobes

The human gut harbors tons of microbes including bacteria, fungi, protozoa, and viruses, collectively known as the gut microbiota. These gut microbiota has established a symbiotic relationship with the host and help the host in metabolism, immune response and homeostasis as well as protection from pathogens. A dysbiosis or imbalance in the gut microbiota environment can cause esophageal or colorectal cancer, colitis and other gut disorders. Strategies to treat gut dysbiosis include fecal microbiota transplant, probiotics or a dietary intervention. Several studies show the potential of phytochemicals in improving this gut microbiota imbalance by selectively killing the pathogenic gut anaerobes and being harmless towards the commensal ones.

We tested the activity of Rom against different gut anaerobes. The MIC values of Rom against different anaerobic strains was done in collaboration with Dr.Lisa Maier from Universitätsklinikum Tübingen, an expert in anaerobic gut microbe research (Table 2). Rom was found to be very active against pathogenic gram positive gut anaerobe *Clostridium difficile* and *Clostridium perfringens* and 2 of the commensal gut anaerobes

Streptococcus parasanguinis and *Bifidobacterium adolescentis.* Rom was inactive against pathogenicgram negative bacteria. Rom was also inactive or very less active against most of the commensal gut anaerobes tested, suggesting its potential as a dietary supplement against gut dysbiosis.

Table 3. MIC of Rom against anaerobic microorganisms

Strains		**Rom MIC (µM)**
Pathogenic G +ve gut anaerobe	*Clostridium difficile*	4
	Clostridium perfringens	4
Commensal gut anaerobe	*Streptococcus salivarius*	<2
	Dorea formicigenerans	22
	Streptococcus parasanguinis	4
	Ruminococcus gnavus	22
	Clostridium ramosum	22
	Blautia obeum	22
	Roseburia intestinalis	22
	Coprococcus comes	22
	Collinsella aerofaciens	22
	Bifidobacterium adolescentis	4
	Parabacteroides distasonis	>45
	Eubacterium rectale	>45
	Lactobacillus paracasei	22
	Clostridium bolteae	>45
	Parabacteroides merdae	22
	Bifidobacterium longum subsp. Longum	>45
	Clostridium saccharolyticum	>45
	Prevotella copri	>45
	Odoribacter splanchnicus	>45
	Fusobacterium nucleatum subsp. Nucleatum	>45
	Bilophila wadsworthia	>45

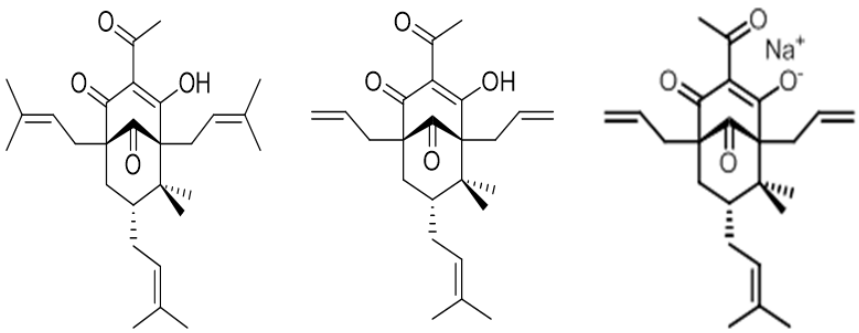
	Bacteroides vulgatus	>45
	Bacteroides uniformis	>45
	Bacteroides thetaiotaomicron	>45
	Bacteroides fragilis NT	>45
Pathogenic G –ve gut anaerobe	*Salmonella enterica* typhimurium TolC	>45
	Yersinia pseudotuberculosis	>45
	Yersinia enterocolitica WA-314	>22
	Vibrio cholerae	>22
	Shigella sonnei 53G	>45
	Shigella flexneri	>45
	Salmonella enterica typhimurium LT2	>45
	Salmonella enterica typhimurium	>45
	Escherichia coli UTI89	>45

Studies on PPAP23 and PPAP53

Synthesis of the sodium salt of PPAP 22, named PPAP 53, has improved solubility

In this paper, we have mainly focused on the activities of PPAP 23, 22 and 53. PPAP 53 is the sodium salt of the newly synthesized PPAP 22. The structures of the three compounds are shown in Figure. 3.14. The difference between PPAP 23 and 22 is that in R2 and R2 of PPAP 23 there are prenyl-residues while in PPAP 22 there are allyl-residues (Guttroff *et al.*, 2017). We thought that the allyl residues in PPAP 22 would increase the solubility a little bit, however, the solubility of PPAP 22 was still comparatively low. Among the tested solvents in which PPAP 22 (5 mg/ml) was soluble were 100% DMSO, 100% ethanol, 80% methanol, 8.2% cyclodextrin/18% DMSO in PBS, and 0.4% Tween 80 / 18% DMSO in PBS. While too high concentrations of DMSO, ethanol and methanol, and beta-cyclodextrin are cytotoxic we have chosen 0.4% Tween 80 / 18% DMSO in PBS as a solvent. To increase the solubility of PPAP 22 its Na-salt, PPAP 53, was synthesized. Comparative *in vitro* determination of MIC values showed that the MIC of PPAP 22 and PPAP 53 was essentially the same for

the multidrug-resistant *S. aureus* USA300, namely ~ 2 µg/ml. For this reason, we used either PPAP 23 or PPAP 53 in our studies. We then moved from *in vitro* studies to *in vivo* studies.



	PPAP 23	PPAP 22	**PPAP 53**
Mass (Dalton)	440,6	384,5	406,5
MIC (µg/ml)	1	4	0.5

Figure 3.14. Structures of PPAP 23, 22 and 53 used in this work. The PPAPs have similar MIC values (1 to 2 µg/ml) for the multi resistant *S. aureus* USA300. PPAP 53 is the Na salt of PPAP 22 and is therefore more water soluble. PPAP 23 was dissolved in DMSO.

PPAP 22 and PPAP 53 had no adverse effect on larvae but could not rescue larvae in an infection model

With PPAP 22 and PPAP 53 we carried out a *Galleria mellonella* larvae infection assays with *S. aureus* USA300. The used dose was 10 to 100 x MIC (20 mg/kg to 200 mg/kg). Without USA300, all larvae survived indicating that PPAP 23 and 53 are well-tolerated at this dose (Figure. 3.15A, B). However, if the larvae were infected with USA300 (10^6 CFU), all larvae died after about 3 days when treated with PPAP 23 (Figure. 3.15. A), and they also couldn't be rescued with the water-soluble PPAP 53 at a dose of 20 mg/kg (50 µM) (Figure. 3.15B). As a control we also tested vancomycin (Van) at a comparable dose 20 mg/kg Van (13 µM). Van could fully protect the USA300 infected larvae (Figure. 3.15C). Since in vitro PPAP 23 and PPAP 53 showed comparable good antimicrobial activity, the question arose why in the infection model the PPAPs were not effective.

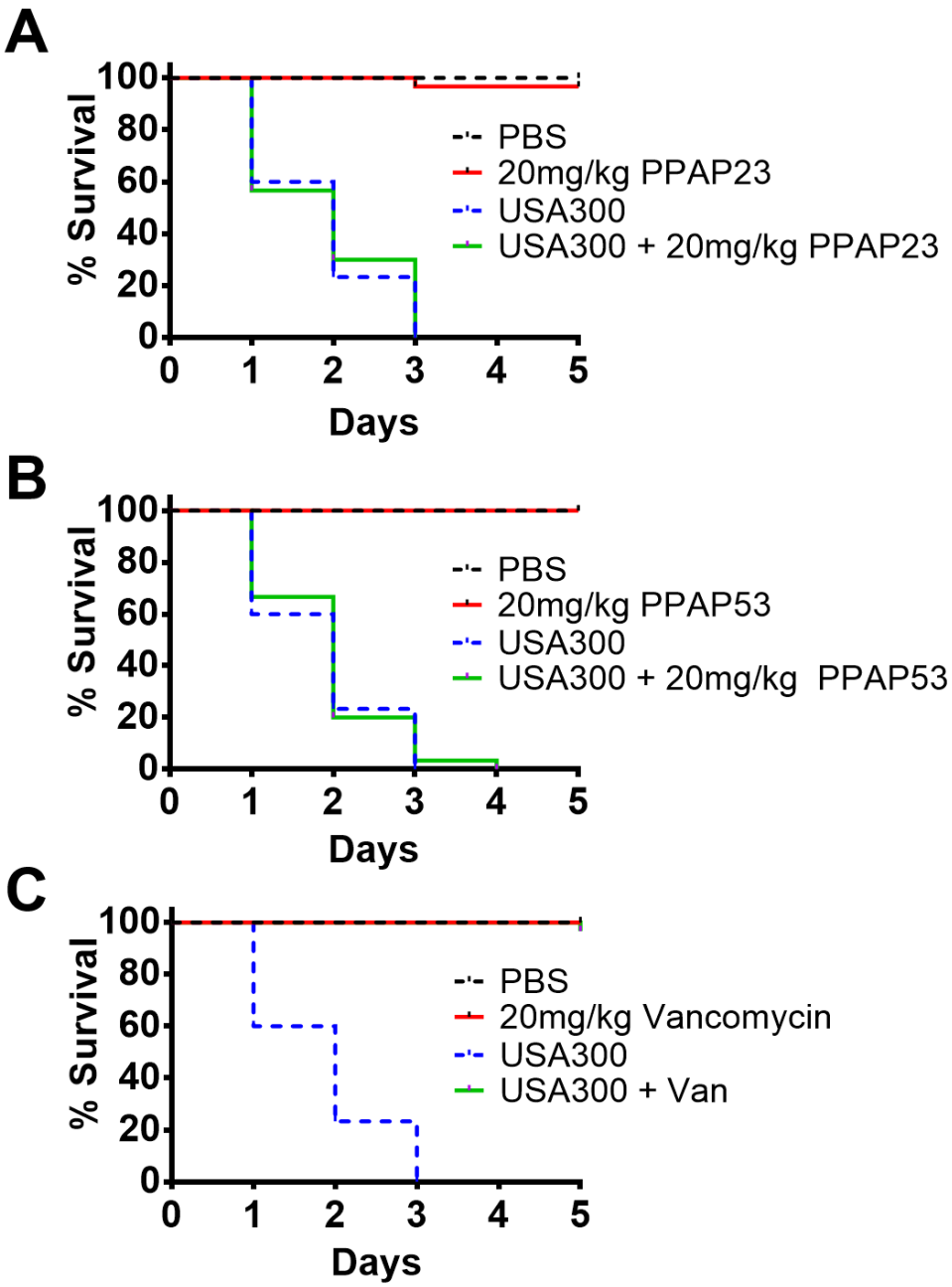


Figure 3.15. PPAP 23 and PPAP 53 are non-toxic to larvae, but failed to protect larvae from infection with *S. aureus* USA300. Ten *Galleria mellonella* larvae per group, with weight average of 500 mg/larvae, were either non-treated, injected with 10^6 colony forming units (cfu) *S. aureus* USA300 (last right proleg). 1 h after administration of bacteria larvae were treated with 20 mg/kg (45 µM) PPAP 23 (**A**), or 20 mg/kg (50 µM) PPAP 53 (**B**), or vancomycin (**C**) at a comparable dose (20 mg/kg, 13 µM). Infected larvae without treatment were normally killed by *S. aureus* after 3 days. The larvae were maintained at 37 °C and observed for mortality every day over the course of 5 days. A total of three biological replicates are represented in the graph.

PPAP 23 showed a beneficial but not full protective effect on *S. aureus* septic arthritis mouse model

Since larval and mammalian models do not always give the same results, we investigated whether PPAP 23 has an effect in the mouse model of septic arthritis caused by *S. aureus* Newman. PPAP 23 was administered at a dose of 100 µg PPAP 23/mouse twice daily starting on day 2 after infection with *S. aureus*. Signs of septic arthritis appeared on day 2 post infection and continued to worsen through day 7. The clinical severity of septic arthritis was similar in PPAP 23-treated mice compared with control mice (Figure. 3.16A). Percent weight loss is a useful parameter for monitoring the severity of systemic staphylococcal infections. Mice treated with either vehicle (0.5% Tween 80 in PBS) or PPAP23 lost up to 25% of their body weight during the experimental period (Figure. 3.16B). No significant difference was observed between the PPAP23 group and controls. Interestingly, however, there were macroscopically more abscesses in the kidneys of mice receiving vehicle compared with PPAP 23-treated mice ($P < 0.05$; Figure. 3.16C). The abscess score and the actual bacterial load in the kidneys were significantly correlated ($r=0.95$; $P<0.001$), and control mice had >25-fold higher bacterial load in the kidneys than PPAP 23-treated mice ($P<0.05$; Figure. 3.16D). This suggests that PPAP 23 treatment had a beneficial effect on bacterial clearance in septic arthritis caused by *S. aureus*.

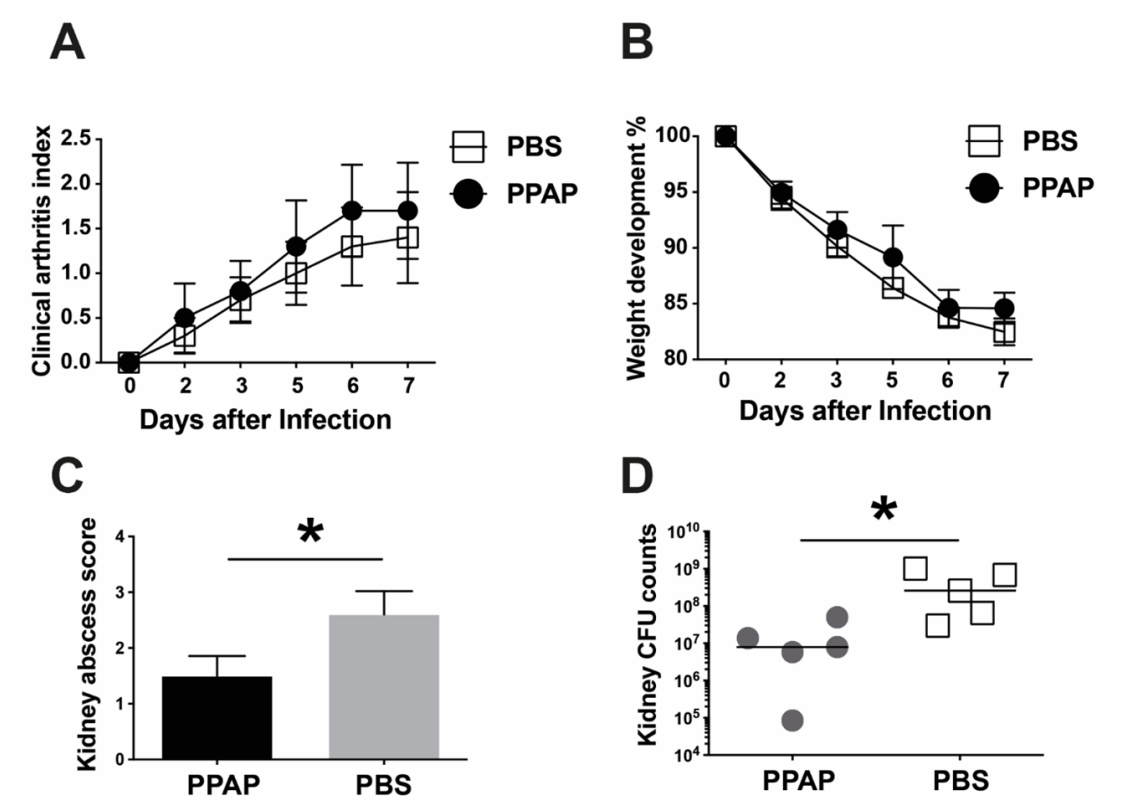


Figure 3.16. PPAP 23 treatment significantly reduced the abscess formation and bacterial load in kidneys in mice with *S. aureus* septic arthritis. NMRI mice inoculated with *S. aureus* Newman strain (4×10^6 colony-forming units/mouse) were treated with PPAP 23 dissolved in 0.5% tween 80 in PBS (100µg/mouse; n = 5) or same volume of 0.5% tween 80 in PBS (n = 5) twice a day starting on day 2 after inoculation with bacteria and continuing until the animals were euthanized on day 7. The severity of clinical arthritis (**A**) and the body weight development (**B**) in the mice were observed for 7 days after infection. Kidney abscess scores (**C**) and persistence of *S. aureus* in kidneys (**D**) from the mice euthanized 7 days after infection. Statistical evaluations were performed using the Mann–Whitney U test. Data are mean values ± standard error of the mean. *P < 0.05.

The coelomic fluid of the larvae antagonized the activity of PPAP 53

We suspected that coelomic fluid abrogated the effect of PPAPs. Therefore, we examined the effect of coelomic fluid on PPAP 53 activity *in vitro*. To mimic the in vivo larval experiment, 100 µl of filter-sterile coelomic fluid of larvae was incubated overnight with 20 mg/kg PPAP 53 and 10^6 CFU/ml *S. aureus* USA300. As a control, PBS was incubated with PPAP 53 and USA300. While PPAP 53 alone was able to kill S. aureus by 3 logs after 24 hours, PPAP 53 failed to kill *S. aureus* in the presence of coelomic fluid (Figure. 3.17A). This result indicates that components in the coelomic fluid have a neutralizing effect on PPAP 53. This result raises the question of whether

components of mammalian blood, like albumin, can also antagonize the activity of PPAP 53.

Bovine serum and albumin neutralized the activity of PPAP 53

Larvae contain hemolymph (coelomic fluid) that is analogous to mammalian blood (Fredrick & Ravichandran, 2012). Since coelomic fluid could antagonize the antimicrobial activity of PPAP 53, we wondered if mammalian serum would have the same effect on PPAP 53 activity. Therefore, the MIC of PPAP 53 was determined in the presence of fetal bovine serum (FBS). We found that 25% FBS decreased the antibacterial activity of PPAP 53 by 32-fold (Table 4). Albumin is the most abundant serum protein, accounting for 55% of all proteins (≈ 35-50 mg/ml), and the second most abundant proteins are immunoglobulins (≈ 10-15 mg/ml) (Anderson & Anderson, 2002, Schuster *et al.*, 2014). Therefore, we investigated whether albumin immunoglobulins can abrogate the antimicrobial activity of PPAP 53 against *S. aureus* USA300. Indeed, whole serum (FBS) and also BSA (50 mg/ml, as found in blood) significantly decreased the antimicrobial activity of PPAP 53 *in vitro* (Figure. 3.17B).

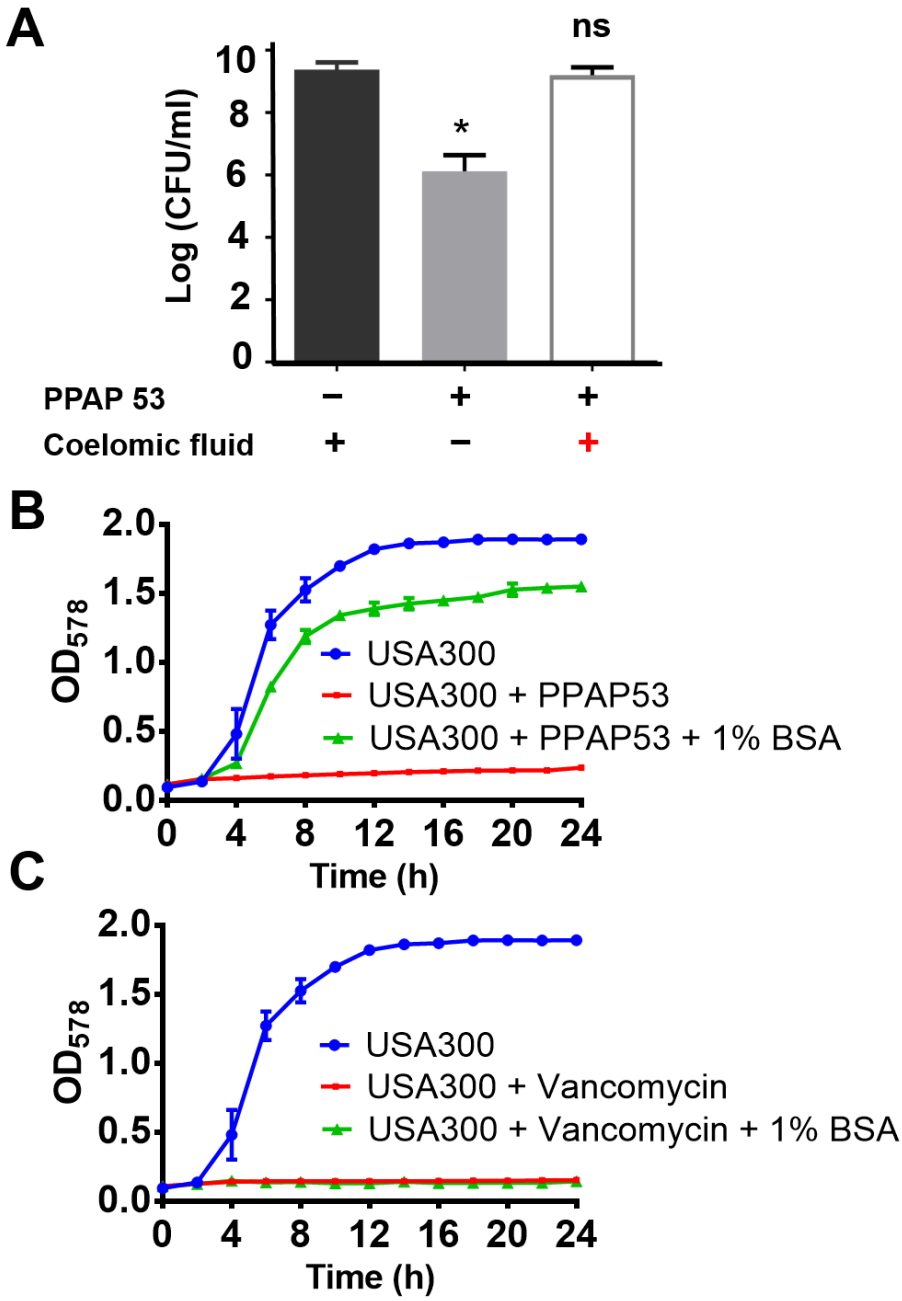


Figure 3.17. The bactericidal activity of PPAP53 is reversed by the larval coelomic fluid or 1% BSA. (A) To mimic *the in vivo* larva infection assay, the *ex vivo* killing assay was adopted. Bacterial inoculum of 10^5 CFU and 10 µg of PPAP was added to 100 µl of larva coelomic fluid as the treatment group. Untreated larval liquid and larval liquid treated with PBS were used as controls. The viability of bacteria in each group was determined by the drop plate method The bactericidal effect of PPAP 53 on *S. aureus* is reversed by coelomic fluid. (B) The bactericidal effect of PPAP 53 (MIC: 0.5-1µg/ml) on *S. aureus* USA300 is reversed by 1% BSA whereas the effect of vancomycin remained unchanged.

Table 4. Impact of serum components on PPAP53 activity

Medium	MIC (µg/ml)	
	PPAP53	Vancomycin
MHB	1	1
MHB + 25% FBS	32	2
MHB + 0.5% BSA	4	1
MHB +1% BSA	8	1
MHB + 2.5% BSA	16	1
MHB + 5% BSA	32	2
MHB + 1% IgG	1	1
MHB + 2.5% IgG	1	1
MHB + 1% Fg	1	1
MHB + 2.5% Fg	1	1

We also investigated the effect of immunoglobulins (IgG) and fibrinogen (Fg), other relatively abundant proteins in serum. However, these serum proteins at concentrations up to 25 mg/ml had no effect on the activity of PPAP 53 (Table 4). It appears that albumin is the major protein in serum that neutralizes the activity of PPAP 53. Studies on the growth kinetics of USA300 in combination with PPAP53 and/or 1% bovine serum albumin (BSA) showed that the bactericidal effect of PPAP 53 was already abolished by 1% BSA. While the bactericidal effect of vancomycin remained unchanged in the presence of 1% BSA (Figure. 3.17C).

In silico docking studies showed PPAP53 binds to the Heme binding pocket of BSA

Serum albumin, the most abundant plasma protein (~640 µM), can bind a wide variety of hydrophobic ligands including fatty acids, bilirubin, thyroxine or hemin (Zunszain *et al.*, 2003). If one knew whether the binding site of PPAP 53 overlapped with one of the known ligands, one could try to displace the binding of PPAP by the corresponding ligand. For this reason, we attempted to identify in silico the binding pocket for PPAP 53. A well-defined crystal structure of bovine serum albumin (BSA) was downloaded

from protein data bank (PDB ID: 3V03). The calcium and acetate ions attached to the crystal structure, as well as the water molecules used for crystallization were removed to get a clean structure of the BSA protein. From the 2D structure of PPAP 53 (Wang *et al.*, 2019), a 3D structure was prepared by Chem3D 16.0 and its geometry was optimised by energy minimisation.

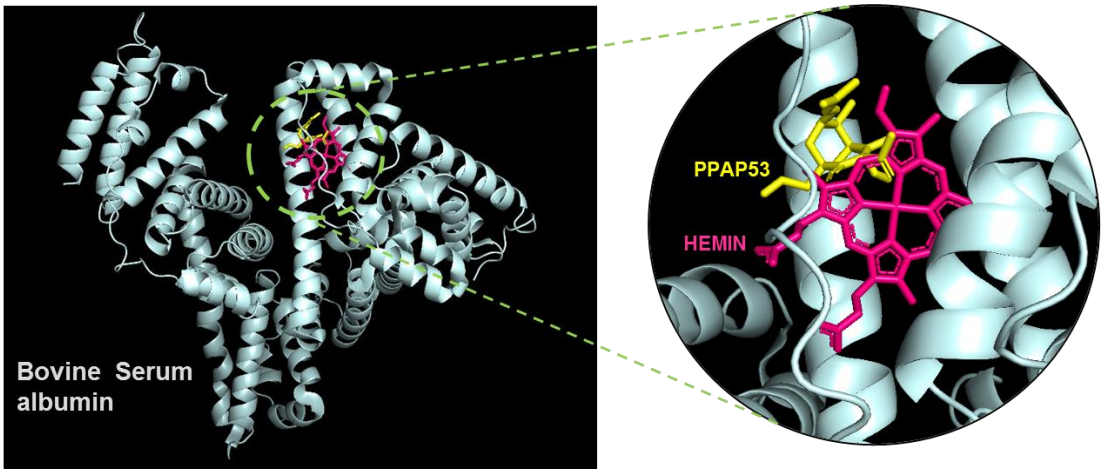


Figure 3.18. PPAP53 binds to the hemin binding pocket of BSA**.** *In silico* docking analysis by AutoDock vina showed that PPAP53 binds to FA1-IB pocket of BSA with a binding energy of -7.2 kcal/mol. This pocket represents the third main ligand (e.g., drug) binding pocket of BSA, the hemin being a prototypical ligand.

The structures were visualized and verified using PyMoL software. The PDBQT files of BSA and ligands were prepared using AutoDock Tools and blind docking was performed in AutoDock vina. During the preparation of PDBQT files polar hydrogens and Kollman charges are added for both the protein and ligand. BSA was considered as a rigid structure and PPAP 53 as the flexible one during docking. *In silico* docking analysis showed that PPAP 53 binds to the IB pocket of BSA with a binding energy of -7.2 kcal/mol (Figure. 3.18). This pocket represents the third main ligand (e.g., drug) binding pocket of BSA, the hemin being a prototypical ligand besides other compounds such as azapropazone, indomethacin and 3, 5-triiodobenzoic acid (Ghuman *et al.*, 2005).

Addition of known BSA ligands couldn't improve the *in vitro* bactericidal activity of PPAP53 in presence of BSA

Addition of known FDA approved drugs that binds to HSA, failed to restore the bactericidal activity of PPAP23. Different drugs/ligands used as well as their binding sites (Ghuman *et al.*, 2005) are given in Table 5. Even though, *in silico* studies showed the binding of PPAP53 at the IB site, same like that of hemin, the addition of hemin, didn't improve the *in vitro* bactericidal activity of PPAP53 (Figure. 3.19A). Figure 19, 20, and 21 shows the growth curve kinetics of USA300 in combination with 1% BSA and its different ligands. The addition of different ligands couldn't resuscitate the bactericidal activity of PPAP53.

Table 5. Tested ligands (FDA approved drugs) binding to HSA and their binding pockets

No.	Drugs tested	Binding pocket of HSA
1	Diffusinal	IIIA, IIA-IIB
2	Iodipamide	IIA, Cleft
3	Azapropazone	IIA, IB
4	Ibuprofen	IIIA, IIA-IIB
5	Diazepam	IIIA
6	Indomethacin	IIA, IB
7	Oxyphenbutazone	IIA, IIIB
8	Phenylbutazone	IIA
9	Warfarin	IIA
10	3,5 diidosalicylic acid	IIA
11	Hemin	IB

PPAP23 is active against pathogenic Gram-positive gut anaerobes

PPAP 23 is already known from our previous publication where we could show that it has good antimicrobial properties against aerobic and facultative aerobic Gram-positive bacteria (Wang *et al.*, 2019). What has not been studied, however, is its effect on anaerobic gut bacteria. Here we show that PPAP 23 is very active against Gram-

positive anaerobic pathogenic gut bacteria such as *Clostridium difficile* and *Clostridium perfringens*. It was also active against some of the commensal gut anaerobes like *Streptococcus salivarius, Ruminococcus gnavus, Clostridium ramosum, Blautia obeum* and *Parabacteroides distasonis.* But it was inactive or very less active against most of the commensal gut anaerobes tested (Table 6). As expected, it was inactive against Gram (-) gut bacteria. These results show that PPAP 23 has the potential to be used as a nutritional supplement in cases of intestinal dysbiosis by *Clostridium difficile*. PPAP 23 would have the advantage over conventional antibiotics that the entire intestinal flora would not be affected.

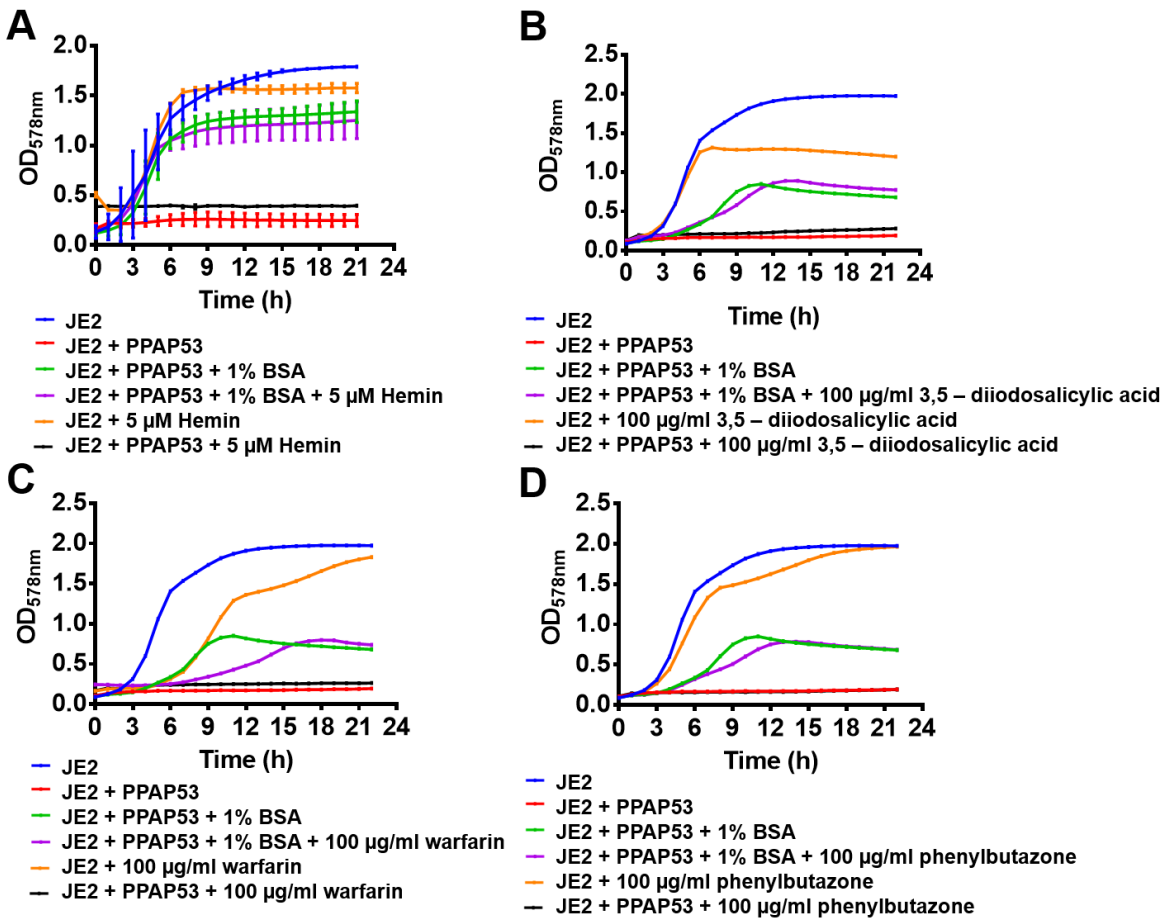


Figure 3.19. Addition of known BSA ligands couldn't improve the in vitro bactericidal activity of PPAP53 in presence of BSA. *S. aureus* precultured in TSB overnight were inoculated to OD=0.01 to a 48 well plate and 1X MIC PPAP53 and/or 1% BSA with or without ligands were added to study effect of peptides on bacterial growth using Varioskan LUX Multimode Microplate Reader. Here with this instrument, a kinetic measurement of optical density 578 nm was obtained every 1 h for a total of 24 hr, at 37°C with continuous shaking. Ligands tested were (A) Hemin, (B) 3,5 diidosalicylic acid (C) warfarin and (D) phenylbutazone.

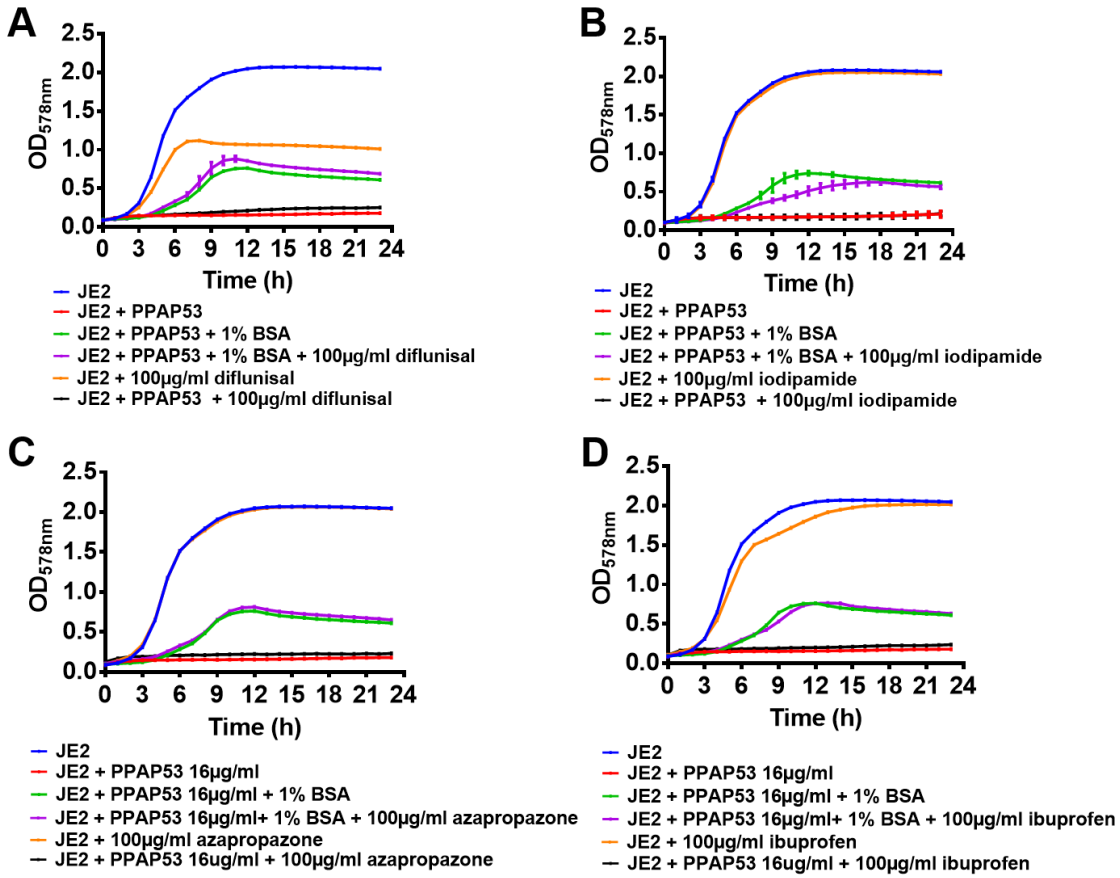


Figure 3.20. Addition of known BSA ligands couldn't improve the in vitro bactericidal activity of PPAP53 in presence of BSA. *S. aureus* precultured in TSB overnight were inoculated to OD=0.01 to a 48 well plate and 1X MIC PPAP53 and/or 1% BSA with or without ligands were added to study effect of peptides on bacterial growth using Varioskan LUX Multimode Microplate Reader. Here with this instrument, a kinetic measurement of optical density 578 nm was obtained every 1 h for a total of 24 hr, at 37°C with continuous shaking. Ligands tested were (A) diffusinal, (B) iodipamide (C) azapropazone and (D) ibuprofen.

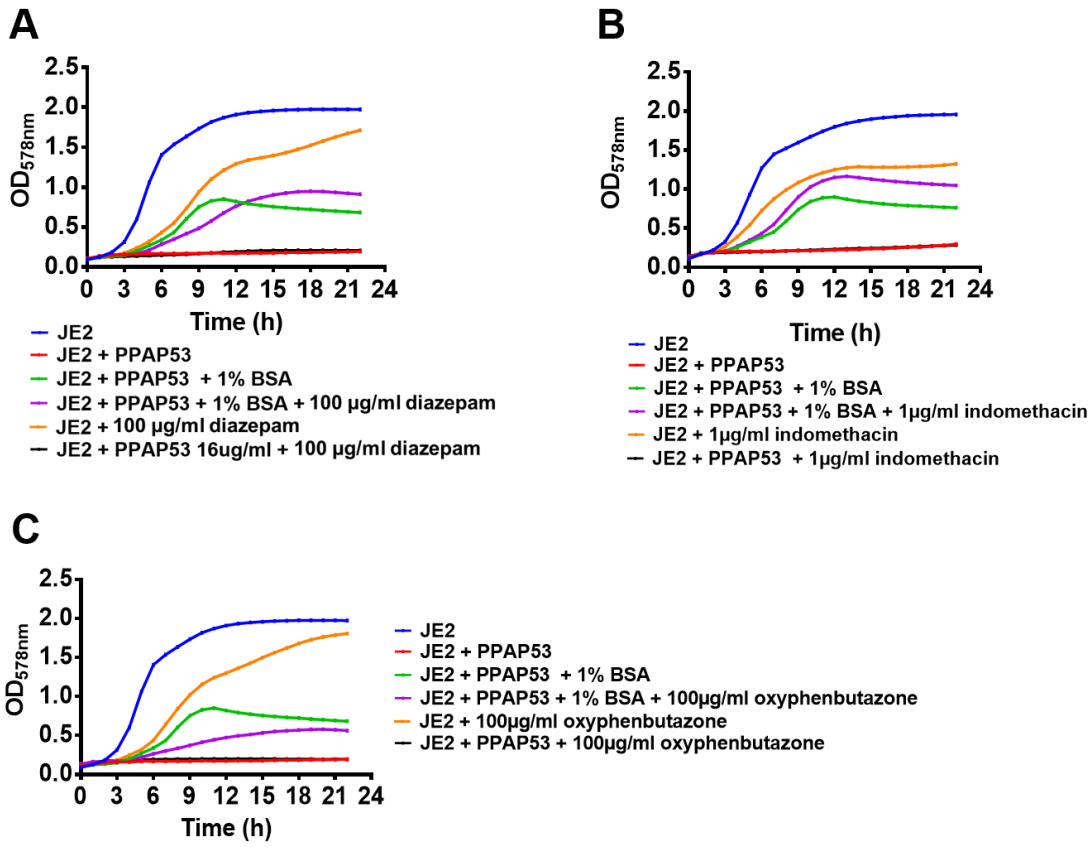


Figure 3.21. Addition of known BSA ligands couldn't improve the in vitro bactericidal activity of PPAP53 in presence of BSA. *S. aureus* precultured in TSB overnight were inoculated to OD=0.01 to a 48 well plate and 1X MIC PPAP53 and/or 1% BSA with or without ligands were added to study effect of peptides on bacterial growth using Varioskan LUX Multimode Microplate Reader. Here with this instrument, a kinetic measurement of optical density 578 nm was obtained every 1 h for a total of 24 hr, at 37°C with continuous shaking. Ligands tested were (A) diazepam (B) indomethacin (C) oxyphenbutazone.

Table 6. MIC values of PPAP23 against some of the anaerobic bacterial strains

Strains		PPAP MIC (μM)
Pathogenic G +ve gut anaerobe	*Clostridium difficile*	<2
	Clostridium perfringens	4
Commensal gut anaerobe	*Streptococcus salivarius*	4
	Dorea formicigenerans	22
	Streptococcus parasanguinis	22
	Ruminococcus gnavus	4
	Clostridium ramosum	4
	Blautia obeum	4
	Roseburia intestinalis	22
	Coprococcus comes	22
	Collinsella aerofaciens	22
	Bifidobacterium adolescentis	45
	Parabacteroides distasonis	4
	Eubacterium rectale	22
	Lactobacillus paracasei	>45
	Clostridium bolteae	22
	Parabacteroides merdae	>45
	Bifidobacterium longum subsp. Longum	22
	Clostridium saccharolyticum	>45
	Prevotella copri	>45
	Odoribacter splanchnicus	>45
	Fusobacterium nucleatum subsp. Nucleatum	>45
	Bilophila wadsworthia	>45
	Bacteroides vulgatus	>45
	Bacteroides uniformis	>45
	Bacteroides thetaiotaomicron	>45
	Bacteroides fragilis NT	>45
Pathogenic G – ve gut anaerobe	*Salmonella enterica* typhimurium TolC	>45
	Yersinia pseudotuberculosis	>22
	Yersinia enterocolitica WA-314	>45
	Vibrio cholerae	>45
	Shigella sonnei 53G	>45
	Shigella flexneri	>45
	Salmonella enterica typhimurium LT2	>45
	Salmonella enterica typhimurium	>45
	Escherichia coli UTI89	>45

Chapter 4

Discussion

Discussion

Heat Shock Protein 90 (Hsp90) is primarily found in the cytoplasmic and nuclear compartments of eukaryotic cells, but it can also associate with the cell membrane under certain conditions. The exact location of Hsp90 on the cell membrane can vary depending on the cell type, the presence of other proteins, and the physiological state of the cell (Hoter *et al.*, 2018, Jackson, 2013).

In some cases, Hsp90 can bind directly to the cell membrane through interactions with membrane-associated proteins, such as lipid rafts or lipid kinases. This can regulate its function and stability, and also allow Hsp90 to interact with transmembrane proteins and modulate their function. Lipid kinases are enzymes that phosphorylate lipids and modify the lipid composition of the cell membrane. Hsp90 can interact with lipid kinases, such as protein kinase C, and modulate their activity. This can have an effect on cell signaling and other cellular processes, such as cell survival and proliferation. Lipid rafts are specialized regions of the cell membrane that are enriched in certain lipids, such as cholesterol and sphingolipids. Lipid rafts play a role in cell signaling, membrane trafficking, and other cellular processes. Hsp90 can also bind to lipid rafts through interactions with raft-associated proteins, such as caveolin.

In other cases, Hsp90 can be associated with the cell membrane indirectly through its interactions with other chaperone proteins, such as Hsp70 or Hsp40, which can recruit Hsp90 to the membrane. Overall, while Hsp90 is primarily found in the cytoplasm and nucleus, its ability to associate with the cell membrane under certain conditions highlights its versatility and dynamic nature as a chaperone protein (Doyle, 2019).

More importantly, the greater wax moth (*Galleria mellonella*) does express Heat Shock Protein 90 (Hsp90). Hsp90 is a conserved chaperone protein found in a wide range of organisms, including insects (Wrońska, 2020).

In *Galleria mellonella*, Hsp90 has been implicated in various biological processes, such as development, stress response, and immunity. For example, Hsp90 has been shown to play a role in the regulation of heat shock response, the regulation of larval-pupal metamorphosis, and the modulation of immune response to fungal infections.

Hsp90 is a heat shock protein that plays a role in regulating larval metamorphosis. It helps modulate the activity of signaling pathways that control developmental transitions. Hsp90 can also regulate the stability of key transcription factors that drive the metamorphic process. In some species, Hsp90 inhibitors have been shown to disrupt the normal progression of metamorphosis. This highlights the important role that Hsp90 plays in controlling this process (Tachibana, 2015).

Hsp90 plays a role in stress regulation in larvae. As a heat shock protein, Hsp90 helps cells cope with environmental stressors such as high temperatures, oxidative stress, and changes in osmotic pressure. Hsp90 acts by stabilizing key regulatory proteins and preventing their degradation, allowing cells to respond effectively to stress. Additionally, Hsp90 can modulate the activity of signaling pathways that control the stress response, further strengthening the cellular response to stress. In some species, Hsp90 inhibitors have been shown to disrupt the stress response in larvae, highlighting the important role that Hsp90 plays in regulating the stress response in this life stage. Overall, the expression of Hsp90 in Galleria mellonella highlights its importance as a conserved chaperone protein across different species and its role in a wide range of biological processes (Sanchez *et al.*, 2020).

Hsp90 and related heat shock proteins are also involved in host defense (Calderwood *et al.*, 2016). For example, GRP94 is primarily found in the endoplasmic reticulum, where it functions as a chaperone for transmembrane proteins and other proteins involved in protein folding and maturation. Hsp90, on the other hand, is found in the cytoplasm and is involved in the folding and stabilization of a wide range of client proteins, including kinases, transcription factors, and other signaling molecules.

In terms of pharmacological targeting, both GRP94 and Hsp90 are potential targets for cancer therapy, GRP94 inhibitors were not yet developed, Hsp90 inhibitors such as geldanamycin are currently in clinical trials (Schaiff *et al.*, 1992, Randow & Seed, 2001, Staron *et al.*, 2010, Mesquita *et al.*, 2017). In fact, GRP94 (GP96) is the master chaperone for Toll-like receptors and is significant in the macrophage innate function (Yang *et al.*, 2007).

F-actin is a component of the cytoskeleton in eukaryotic cells and plays an important role in various cellular processes such as cell migration, cell division, and cell shape maintenance. The following can be signaling pathways involved in regulating F-actin dynamics and more studies need to be done to identify the same:

(i) Rho GTPase signaling pathway - Activation of Rho family GTPases, such as RhoA, leads to the activation of downstream effectors, including Rho-associated kinase (ROCK) and myosin light chain kinase (MLCK), which in turn regulate the assembly and disassembly of F-actin. (ii) Rac/Cdc42 signaling pathway - The small GTPases Rac1 and Cdc42 play a role in actin polymerization and filopodia formation by regulating actin nucleation and branching. (iii) WASp/Arp2/3 complex signaling pathway - The WASp/Arp2/3 complex is involved in actin nucleation and branching, leading to the formation of new actin filaments. (iv) Integrin signaling pathway - Integrins are transmembrane proteins that link the extracellular matrix to the cytoskeleton. Integrin signaling regulates F-actin organization and cell adhesion. (v) Phosphoinositide 3-kinase (PI3K)/Akt signaling pathway - PI3K-Akt signaling regulates actin polymerization and cell migration by controlling the activity of Rho family GTPases and other cytoskeleton-regulating proteins.

Polycyclic polyprenylated acylphloroglucinols (PPAPs) are a class of compounds found in plants and are known for their diverse biological activities, including antioxidant, anti-inflammatory, and anti-tumor properties (Ciochina & Grossman, 2006, Richard *et al.*, 2012, Yang *et al.*, 2018, Bailly & Vergoten, 2021). PPAPs are derived from polyprenylated phloroglucinols and are unique in their molecular structure, which includes both prenyl and acyl groups. This unique structure gives PPAPs the ability to act as antioxidants by scavenging free radicals and reducing oxidative stress, as well as exhibiting anti-inflammatory effects by suppressing the activation of immune cells (Cao *et al.*, 2020, Fromentin *et al.*, 2015, Coste *et al.*, 2020, Ma *et al.*, 2021). Additionally, PPAPs have shown anti-tumor effects in some studies, making them promising candidates for the development of new cancer therapies. Even though PPAPs have demonstrated anti-cancer activities in some studies, but the exact mechanisms of action are not fully understood. Here are some potential modes of action that have been proposed: (i) Antioxidant effects: PPAPs have strong antioxidant properties, and it is believed that their ability to scavenge free radicals and reduce

oxidative stress may help prevent the formation of cancer cells or slow their growth. (ii) Anti-inflammatory effects: PPAPs have shown anti-inflammatory effects, and it is believed that their ability to suppress the activation of immune cells may play a role in their anti-cancer activities. (iii) Apoptosis induction: Some studies have shown that PPAPs can induce apoptosis (programmed cell death) in cancer cells, which can lead to their death and slow the progression of the disease. (iv) Inhibition of angiogenesis: PPAPs have been shown to inhibit angiogenesis, the process by which new blood vessels are formed, which is critical for the growth and spread of cancer (Bailly & Vergoten, 2021, Han *et al.*, 2020).

In collaboration with the research group of Bernd Plietker (Oganic Chemistry, University of Dresden), we are concerned with the antimicrobial properties of novel synthetic type B PPAP-derivatives (Biber *et al.*, 2011, Horeischi *et al.*, 2014). Here we compared two PPAP derivatives, PPAP 23 and PPAP 53, a more water-soluble sodium salt of PPAP22. We primarily used the multidrug-resistant *S. aureus* USA300 as the pathogenic test strain. The mode of action of PPAP 23 has been described already earlier (Guttroff *et al.*, 2017, Wang *et al.*, 2019). While in previous studies we were mainly concerned with the *in vitro* antimicrobial activity, we have focused here on the *in vivo* effect. Since PPAP 53 is a new compound, we first carried out comparative studies in vitro. Surprisingly, we found that the Na salt of PPAP 53 exhibited *in vitro* almost the same good antimicrobial properties (MIC ~ 2 µg/ml) as the less water-soluble PPAP 23. We then extended our studies to the wax moth model. Here, both compounds were found to be well tolerated at a dose up to 200 mg/kg (100 x MIC) and no larvae died in the 5-day period. Infecting the larvae with a dose 10^6 CFU USA300 they died within 3 days. Neither PPAP 23 nor PPAP 53 at a dose of 10 x MIC (20 mg/kg) could save the larvae, but vancomycin at a comparable dose could.

Since one cannot necessarily infer the same results from an insect model in a mammalian model, we tested PPAP 23 in a septic arthritis mouse model. As common practice with antibiotics, PPAP23 was administered twice daily at a dose of 100 µg/mouse. The first treatment was given 2 days after infection with *S. aureus* Newman. PPAP23 failed to prevent the progression of septic arthritis. However, it caused a reduction in abscess formation in the kidneys and the bacterial load was also decreased (Figure. 3.16). From this we see that PPAP23 cannot stop an incipient

septic arthritis. It follows that PPAP23 cannot stop an incipient septic arthritis, but it can reduce the bacterial load somewhat most likely by its antibiotic effect.

What concerned us most, however, was the question why the two PPAPs worked so well *in vitro* but largely failed to work *in vivo*. The answer was that not only larval coelomic fluid but also bovine serum and albumin (BSA) neutralized the activity of PPAP 53 (Figure. 3.17). Other very common serum proteins such as immunoglobulins or fibrinogen had no neutralizing effect (Table 4).

Serum albumin is a protein that is found in the blood and is the most abundant protein in the plasma. It plays important roles in maintaining blood volume, regulating blood pressure, transporting hormones, and binding and neutralizing drugs and other substances. Serum albumin is produced by the liver and is commonly used as a biomarker for liver function and overall health. Low levels of serum albumin can indicate liver disease, malnutrition, and other health conditions. The binding of drugs to serum albumin in the blood can lead to their neutralization, affecting their pharmacokinetics and efficacy. This occurs due to the high binding affinity of albumin for certain substances, including drugs. The binding of drugs to albumin can trap the drugs in circulation, prevent their passage into tissues, and alter their distribution, elimination, and overall effectiveness. This process of drug neutralization by serum albumin is a significant factor in drug pharmacology and can impact the efficacy and toxicity of drugs in the body (Peters Jr, 1995).

Hyperforin has been shown to bind to serum albumin, the most abundant protein in the blood plasma, and to other plasma proteins. The binding of hyperforin to albumin and other plasma proteins can affect its pharmacokinetics, including its distribution, elimination, and overall effectiveness (Fuller *et al.*, 2018, Traeger *et al.*, 2020). The binding of hyperforin to albumin can also alter its bioavailability, and the extent of binding can vary depending on the dose and duration of administration. Understanding the binding of hyperforin to plasma proteins is important for the development of effective and safe therapeutic strategies for its use. There is currently no clear way to prevent the binding of hyperforin to albumin. However, some strategies that may reduce the binding of hyperforin to albumin include: (i) Formulation design: Modifying the formulation of hyperforin, such as encapsulating it in liposomes or nanoparticles,

may reduce its binding to albumin and increase its bioavailability (Traeger *et al.*, 2020). (ii) Administration route: Changing the administration route of hyperforin, such as from oral to transdermal, may reduce its binding to albumin and increase its bioavailability. (iii) Co-administration with other compounds: Co-administering hyperforin with other compounds that have been shown to reduce its binding to albumin, such as ascorbic acid, may also help reduce its binding and increase its bioavailability.

We thought of a similar strategy namely to identify the binding site of PPAP to albumin by in silico docking and to suppress the binding of PPAP by ligands that bind to the same site. We found that PPAP53 binds to the heme-binding pocket of BSA, which is also the binding site of several other compounds. However, all of the different ligands that we tested (Table 5) failed to restore the bactericidal activity of PPAP23. This could mean that PPAP 53 also binds to other sites of albumin, or that the binding of PPAP 53 to albumin is stronger than that of the other tested ligands. We assume that the latter is the case. These results show that PPAP 23 and PPAP 53 are of limited use in the treatment of septic infections because of the high albumin content in the blood.

However, could these PPAPs play a role in the treatment of gastrointestinal infections? An examination of plasma proteins in the faeces of healthy volunteers showed that the main plasma proteins, albumin and IgG, were detectable but only in traces. When albumin was added to the faeces, it was rapidly degraded within a few hours (Schmidt *et al.*, 1995). The albumin concentration in humans is 0.01-0.24 mg/g wet weight of faeces (Morrow *et al.*, 1990). At this low albumin concentration in the gastrointestinal tract (GIT), the antimicrobial activity of PPAP 23 and PPAP 53 is unlikely to be affected. Furthermore, in patients with inflammatory bowel disease and ulcerative colitis, the albumin levels are so low due to the protein loss from inflammation and diarrhea, (Su *et al.*, 2019, Wang *et al.*, 2022), suggesting that the albumin content in GIT might also be decreased.

Considering the possibility that PPAP 23 and PPAP 53 may have an effect in GIT infections, we investigated the antimicrobial activity of typical pathogenic and commensal anaerobic GIT bacteria. Interestingly, some of the most feared intestinal pathogenic bacteria are particularly susceptible to PPAP 53. These include *Clostridium difficile* and *Clostridium* perfringens, well-known intestinal pathogens the one causing

colitis (Abt *et al.*, 2016) the other causes food poisoning and antibiotic-associated diarrhea (Eichner *et al.*, 2017). *Clostridium ramosum* is occasionally found to cause bacteraemia (Legaria *et al.*, 2020). *Parabacteroides distasonis* is an aerotolerant gut anaerobe with emerging antimicrobial resistance and pathogenic and probiotic roles in human health (Ezeji *et al.*, 2021). *Ruminococcus gnavus*, a member of the human gut microbiome associated with Crohn's disease, produces an inflammatory polysaccharide (Henke *et al.*, 2019). The majority of the commensal Gram-positive gut anaerobes and also Gram-negative bacteria are not affected by PPAP 53.

Conclusion

Compounds that interact with Hsp90 and affect cellular physiology are particularly noteworthy. The Lpl1-derived small peptides, L15 and L13, not only alter the cytoskeleton and the associated internalization of S. aureus by host cells in vitro, but also demonstrate a global effect on the entire organism in both insect and mammalian models by decreasing the lethality of S. aureus infection. The exact mechanism for this is still uncertain. Currently, two possibilities are being considered: a) binding of the peptides to Hsp90 and related Hsp proteins activates the innate immune system in a way that it responds more quickly or effectively to S. aureus infection, or b) binding of the peptides to Hsp90 interferes with the binding of the membrane-anchored Lpl proteins of *S. aureus*. Both mechanisms may contribute to reducing the lethality of a *S. aureus* infection.

The lipidomic analysis gives us with a solid proof that in RomR strains, the antibacterial activity of Rom is nullified by the neutralising effect of phospholipids. We could see significantly higher amount of phospholipids excreted by the RomR strains in comparison to the wild type. In RomR strain, FarE, functioning as the effux pump is overexpressed. This overexpression leads to high accumulation of phospholipids in the bacterial supernatant and cell envelope. These accumulated phospholipids neutralize rom in RomR strains. Additionally, potassium salt of Rom, FH-54 was found to be the best candidate among all the derivatives tested, considering it is active even against the RomR strains. Also, Rom was seen to be active against pathogenic gut anaerobes, which gives another potential applications for Rom.

Here we show that PPAP 23 and PPAP 53 are only conditionally suitable for therapeutic use in Gram-positive sepsis since the high albumin co-centration in the blood abrogates their antimicrobial effect. However, a possible application in environments with low albumin content, such as in the gastrointestinal tract or topical application in skin infections, is conceivable. Neutralization by albumin seems to be a general problem of the hydrophobic PPAPs. Work should therefore be done to mitigate the hydrophobic character by introducing hydrophilic side groups. Neutralization by albumin seems to be a general problem of the hydrophobic PPAPs. Work should

therefore be done to mitigate the hydrophobic character by introducing hydrophilic side groups. To what extent this will change the diverse activities remains to be seen.

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Appendix

Appendix A: Abbreviations

Ala	Alanine
AMPs	Antimicrobial Peptides
ApoL-III	Apolipophorin III
Arg	Arginine
Atl	Autolysin
BSA	Bovine Serum Albumin
CDC	Centers For Disease Control and Prevention
Cys	Cysteine
Eap	Extracellular Adherence Protein
ECDC	The European Centre for Disease Prevention and Control
FA	Fatty Acid
FarR	Regulator of Fatty Acid Resistance
FDA	Food and Drug Administration
Fe	Iron
FnBPA	Fibronectin Binding Proteins A
FnBPB	Fibronectin Binding Proteins B
FtsZ	Filamenting Temperature Sensitive Mutant Z
Gly	Glycine
GmCP8	Cationic Protein 8
GTP	Guanosine Triphosphate
GTPase	Guanosine Triphosphatase
HaCaT	Human Keratinocyte Cells
HEK	Human Embryonic Kidney Cells
Hla	Human Leukocyte Antigens
Hsp90α	Heat Shock Protein A
Hsp90β	Heat Shock Protein B
HT-29	Human Colorectal Adenocarcinoma Cells
IsdB	Iron-Regulated Surface Determinant B
Leu	Leucine
Lgt	Lipoprotein Diacylglyceryl Transferase
Lnt	Lipoprotein N-Acyl Transferase
Lpl	Lipoprotein Like Lipoprotein
Lpp	Lipoproteins

Lsp	Lipoprotein Signal Peptidase
MDR	Multidrug Resistant
MM6	Human Monocytic Cell Line
Mn	Manganese
Mo	Molybdenum
MRSA	Methicillin-Resistant *Staphylococcus aureus*
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
Ni	Nickel
NlpC/P60	New Lipoprotein C/Protein Of 60-Kda
NPPCs	Non-Professional Phagocytic Cell
PBMC	Peripheral Blood Mononuclear Cell
PDR	Pandrug Resistant
PG	Phospatidylglycerol
PGRPs	Peptidoglycan Recognition Proteins
PPAP	Polycyclic Polyprenylated Acylphloroglucinol
PrsA	Peptidylprolyl Isomerase
Psmβ-1	Proteasome Subunit Beta Type 1
Rom	Rhodomyrtone
Rom^R	Rhodomyrtone Resistance
ROS	Reactive Oxygen Species
SCO	Synthesis Of Cytochrome C Oxidase
Ser	Serine
Sp	Signal Peptide
TLR2	Toll-Like Receptor 2
VISA	Vancomycin Intermediate resistant Staphylococcus aureus
XDR	Extensively Drug Resistant
Zn	Zinc

Symbols

Δ	Genetic deletion
°C	Degree Celsius
%	Percentage
µ	Micro

Appendix B: List of figures

Figure 3.2. L15, L13 and geldanamycin doesn't have any effect on cell lines (A) HaCaT and (B) N/TERT-1 during treatment time. (C) L13, L15 doesn't affect the USa300 growth in TSB at 37 ℃.

Figure 3.3. L15, L13 and geldanamycin interaction with Hsp90α effects the F-actin formation in (A) HaCaT and (B) N/TERT-1 cells. A significant reduction of F-actin levels were observed on treatment of keratinocytes with peptides and geldanamycin. Error bars show standard deviation of the mean of 3 biological replicates. P values were obtained by student's T-test. : * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; and **** $p < 0.0001$

Figure 3.4. L13/L15 is non-toxic to (A) HaCaT, (B) HEK, (C) HT-29 and (D) MM6 cells *in vitro*. Error bars show standard deviation of the mean of 3 biological replicates. P values were obtained by student's T-test. : * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; and **** $p < 0.0001$.

Figure 3.5. L15/L13 rescues larvae from *S.aureus* infection. Each group consists of 10 larvae with weight average of 500mg/larvae, and were administered with bacteria and/or peptides on its last proleg using a BD insulin syringe. Larvae were injected with different concentrations of (A) L15 and (B) L13 to find the optimum dosage against USA300 infection. Each larvae were injected with 10µl of L15 (last left proleg) 1h before injection of 10^6 cfu *S. aureus* (last right proleg) (C) USA300 and (D) Newman. The larvae were maintained at 37 °C and checked for its mortality every day for the study period of 5 days. Total of 3 biological replicates are represented in the graph.

Figure 3.6. L13, L15 doesn't affect the hemolysin activity of USA300

Figure 3.7. L15 treatment reduces systemic *S. aureus* infection. NMRI mice infected intravenously with *S. aureus* Newman strain ($2x10^6$ CFU/mouse) were treated with L15 intraperitoneally (10mg/kg) or PBS for control starting two hours before inoculation and continuing twice daily until animals were euthanized on day 7. (A) Weight development observed during 7 days. (B) Bacterial load in kidneys on day 7 post-infection. (C) Survival graph of mice infected with *S. aureus*.

Figure 3.8. Multiple sequence alignments of Lpl1 from *S. aureus* USA300 with other bacteria. These include *Staphylococcus epidermidis* SE62, *Staphylococcus hyicus* NCTC 8294, *Staphylococcus schweitzeri* NCTC 13712, *Staphylococcus argenteus* B3-

25B, *Listeria monocytogenes* ATCC 15313, *Ligilactobacillus ruminis* ATCC 27780, *Escherichia coli* NCTC 9001, *Klebsiella pneumoniae* NCTC 9633 and *Pseudomonas aeruginosa* PA216. The lipoprotein signal peptide is indicated by the bracket, the conserved core region by the bar and the L15 sequence is boxed.

Figure 3.9. Multiple sequence alignments of Lpl1 – Lpl9.

Figure 3.10. L15/L13 couldn't rescues larvae from (A) *E. coli* K12 and (B) *P. aeruginosa* PA01 infection. Ten *Galleria mellonella*, larvae per group with weight average of 500mg/larvae were injected with bacteria and/or peptides on its last proleg using a BD insulin syringe. Each larvae were injected with 10µl of L15 (last left proleg) 1h before administration of bacteria. The larvae were maintained at 37 °C and observed for its mortality every day for the study period of 5 days. Total of 3 biological replicates are represented in the graph.

Figure 3.11. Cytokine quantification of PBMCs stimulated by L15, showed that the L15 didn't cause any immune stimulation at the tested concentration. Statistic significances were calculated between the peptide treated cells compared to control by using one-way ANOVA analysis with Tukey's multiple comparison test: *p< 0.05, **p<0.01, ***p<0.001, ns > 0.05.

Figure 3.12. (A) Effect of L15 and L13 on S. aureus USA300 phagocytosis by primary human CD14+ monocytes. Influence of L13 and L15 on the response of host innate immune cells were tested. Release of (B) IL-6 and (C) TNF-α in the supernatant of *S. aureus*-infected PBMCs was assayed by ELISA 20 h after stimulation with L13 or L15 or geldanamycin. C indicates control cells without peptide pretreatment. Samples from 4 donors were carried out in triplicate. Samples from 4 donors were carried out in duplicate. Error bars represent SEM. Statistic significances were calculated between the peptide treated cells compared to control by using one-way ANOVA analysis with Tukey's multiple comparison test: *p< 0.05, **p<0.01, ***p<0.001, ns > 0.05.

Figure 3.13. L15/L13 in combination with different TLR ligands (A) MDP, (B) CpG and (C) ssRNA couldn't rescues larvae from *S. aureus* infection. Ten *Galleria mellonella*, larvae per group with weight average of 500mg/larvae were injected with bacteria and/or peptides on its last proleg using a BD insulin syringe. Each larva was injected with 10µl of L15 (last left proleg) 1h before administration of bacteria. The larvae were

maintained at 37 °C and observed for its mortality every day for the study period of 5 days. Total of 3 biological replicates are represented in the graph.

Figure 3.14. Structures of PPAP 23, 22 and 53 used in this work. The PPAPs have similar MIC values (1 to 2 µg/ml) for the multi resistant *S. aureus* USA300. PPAP 53 is the Na salt of PPAP 22 and is therefore more water soluble. PPAP 23 was dissolved in DMSO.

Figure 3.15. PPAP 23 and PPAP 53 are non-toxic to larvae, but failed to protect larvae from infection with *S. aureus* USA300. Ten *Galleria mellonella* larvae per group, with weight average of 500 mg/larvae, were either non-treated, injected with 10^6 colony forming units (cfu) *S. aureus* USA300 (last right proleg). 1 h after administration of bacteria larvae were treated with 20 mg/kg (45 µM) PPAP 23 (**A**), or 20 mg/kg (50 µM) PPAP 53 (**B**), or vancomycin (**C**) at a comparable dose (20 mg/kg, 13 µM). Infected larvae without treatment were normally killed by *S. aureus* after 3 days. The larvae were maintained at 37 °C and observed for mortality every day over the course of 5 days. A total of three biological replicates are represented in the graph.

Figure 3.16. PPAP 23 treatment significantly reduced the abscess formation and bacterial load in kidneys in mice with *S. aureus* septic arthritis. NMRI mice inoculated with *S. aureus* Newman strain (4×10^6 colony-forming units/mouse) were treated with PPAP 23 dissolved in 0.5% tween 80 in PBS (100µg/mouse; n = 5) or same volume of 0.5% tween 80 in PBS (n = 5) twice a day starting on day 2 after inoculation with bacteria and continuing until the animals were euthanized on day 7. The severity of clinical arthritis (**A**) and the body weight development (**B**) in the mice were observed for 7 days after infection. Kidney abscess scores (**C**) and persistence of *S. aureus* in kidneys (**D**) from the mice euthanized 7 days after infection. Statistical evaluations were performed using the Mann–Whitney U test. Data are mean values ± standard error of the mean. *P < 0.05.

Figure 3.17. The bactericidal activity of PPAP53 is reversed by the larval coelomic fluid or 1% BSA. (A) To mimic *the in vivo* larva infection assay, the *ex vivo* killing assay was adopted. Bacterial inoculum of 10^5 CFU and 10 µg of PPAP was added to 100 µl of larva coelomic fluid as the treatment group. Untreated larval liquid and larval liquid treated with PBS were used as controls. The viability of bacteria in each group was determined by the drop plate method. The bactericidal effect of PPAP 53 on *S. aureus*

is reversed by coelomic fluid. (B) The bactericidal effect of PPAP 53 (MIC: 0.5-1µg/ml) on *S. aureus* USA300 is reversed by 1% BSA whereas the effect of vancomycin remained unchanged.

Figure 3.18. PPAP53 binds to the hemin binding pocket of BSA**.** *In silico* docking analysis by AutoDock vina showed that PPAP53 binds to FA1-IB pocket of BSA with a binding energy of -7.2 kcal/mol. This pocket represents the third main ligand (e.g., drug) binding pocket of BSA, the hemin being a prototypical ligand.

Figure 3.19. Addition of known BSA ligands couldn't improve the in vitro bactericidal activity of PPAP53 in presence of BSA. *S. aureus* precultured in TSB overnight were inoculated to OD=0.01 to a 48 well plate and 1X MIC PPAP53 and/or 1% BSA with or without ligands were added to study effect of peptides on bacterial growth using Varioskan LUX Multimode Microplate Reader. Here with this instrument, a kinetic measurement of optical density 578 nm was obtained every 1 h for a total of 24 hr, at 37°C with continuous shaking. Ligands tested were (A) Hemin, (B) 3,5 diidosalicylic acid (C) warfarin and (D) phenylbutazone.

Figure 3.20. Addition of known BSA ligands couldn't improve the in vitro bactericidal activity of PPAP53 in presence of BSA. *S. aureus* precultured in TSB overnight were inoculated to OD=0.01 to a 48 well plate and 1X MIC PPAP53 and/or 1% BSA with or without ligands were added to study effect of peptides on bacterial growth using Varioskan LUX Multimode Microplate Reader. Here with this instrument, a kinetic measurement of optical density 578 nm was obtained every 1 h for a total of 24 hr, at 37°C with continuous shaking. Ligands tested were (A) diffusinal, (B) iodipamide (C) azapropazone and (D) ibuprofen.

Figure 3.21. Addition of known BSA ligands couldn't improve the in vitro bactericidal activity of PPAP53 in presence of BSA. *S. aureus* precultured in TSB overnight were inoculated to OD=0.01 to a 48 well plate and 1X MIC PPAP53 and/or 1% BSA with or without ligands were added to study effect of peptides on bacterial growth using Varioskan LUX Multimode Microplate Reader. Here with this instrument, a kinetic measurement of optical density 578 nm was obtained every 1 h for a total of 24 hr, at 37°C with continuous shaking. Ligands tested were (A) diazepam (B) indomethacin (C) oxyphenbutazone.

Appendix C: List of tables

Appendix D: Publications and personal contributions to publications

Publications discussed in the thesis

1. Li Huang*, Miki Matsuo*, Carlos Calderón*, Sook-Ha Fan, **Aparna Viswanathan Ammanath**, Xiaoqing Fu, Ningna Li, Arif Luqman, Marvin Ullrich, Florian Herrmann, Martin Maier, Anchun Cheng, Fajun Zhang, Filipp Oesterhelt, Michael Lämmerhofer, Friedrich Götz (2022). Molecular Basis of Rhodomyrtone Resistance in *Staphylococcus aureus*. *Mbio*, **13**: e03833-21.

*: Equal contribution

I performed some of the bacterial killing assays and helped in preparation of the manuscript.

2. From a Hsp90 - binding protein to a peptide drug

Aparna Viswanathan Ammanath, Anders Jarneborn, Minh-Thu Nguyen, Laura Wessling, Paula Tribelli, Mulugeta Nega, Christian Beck, Arif Luqman, Khaled A Selim, Hubert Kalbacher, Boris Macek, Sandra Beer Hammer, Tao Jin, Friedrich Götz (2022), From a Hsp90 - binding protein to a peptide drug, *microLife*, uqac023.

I planned and performed most of the experiments. I analyzed all the experimental data and wrote the manuscript under the guidance of Prof. Friedrich Götz.

Publications not discussed in the current thesis

a) Research articles

3. Huang, Li, Mafeng Liu, **Aparna Viswanathan Ammanath**, Dekang Zhu, Renyong Jia, Shun Chen, Xinxin Zhao, Qiao Yang, Ying Wu, Shaqiu Zhang, Juan Huang, Xumin Ou, Sai Mao, Qun Gao, Di Sun, Bin Tian, Friedrich Götz, Mingshu Wang, Anchun Cheng (2021). Identification of the Natural

Transformation Genes in *Riemerella anatipestifer* by Random Transposon Mutagenesis. *Frontiers in microbiology* **12**: 712198.

I performed *in silico* protein structure determination and docking studies, supported the writing and improvements of the article and did critical proofreading of the manuscript

4. Fredrick, R., Podder, A., **Viswanathan, A.**, Bhuniya, S (2019). Synthesis and characterization of polysaccharide hydrogel based on hydrophobic interactions. Journal of Applied *Polymer Science*, **136**: 47665.

I performed the cytotoxicity and haemolysis experiments.

5. Ruth Bright Chirayath, **Aparna Viswanathan A**, R. Jayakumar, Raja Biswas, Lakshmi Sumitra Vijayachandran (2019). Development of Mangifera indica leaf extract incorporated carbopol hydrogel and its antibacterial efficacy against *Staphylococcus aureus*. Colloids and Surfaces B: *Biointerfaces* **178**: 377-384.

I co-performed most of the experiments and helped in manuscript preparation.

b) Review

6. Sankaran Sathianarayanan, **Aparna Viswanathan Ammanath**, Raja Biswas, Anita B, Sunitha Sukumar, Baskar Venkidasamy (2022). A new approach against *Helicobacter pylori* using plants and its constituents: A review study. *Microbial Pathogenesis* **168**: 105594.

I co-wrote the review with other authors.

c) Book chapters

7. **Viswanathan, Aparna**, Jayakumar Rangasamy, Raja Biswas (2019). Functionalized antibacterial nanoparticles for controlling biofilm and intracellular infections. In *Surface Modification of Nanoparticles for Targeted Drug Delivery*, Springer, Cham, 183-206.

This book chapter was written by myself with assistance of Jayakumar Rangasamy, and Raja Biswas

8. **Aparna Viswanathan A**., Raja Biswas, R. Jayakumar (2019). Targeted nanoparticles for treating infectious diseases. In *Biomimetic Nanoengineered Materials for Advanced Drug Delivery*, Elsevier, 169-185.

This book chapter was written by myself with assistance of Jayakumar Rangasamy, and Raja Biswas

9. *In press*: **Aparna Viswanathan Ammanath**, Jolly Thomas, Anjana Jayasree. Hyaluronic Acid in Tissue engineering. In *Natural Biopolymers in Drug Delivery & Tissue Engineering*, Elsevier.

I co-authored this book chapter with other authors.

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3) Applicability of Terms. The Terms govern User's use of Works in connection with the relevant License. In the event of any conflict between General Terms and Order Confirmation Terms, the latter shall govern. User acknowledges that Rightsholders have complete discretion whether to grant any permission, and whether to place any limitations on any grant, and that CCC has no right to supersede or to modify any such discretionary act by a Rightsholder.

4) Representations; Acceptance. By using the Service, User represents and warrants that User has been duly authorized by the User to accept, and hereby does accept, all Terms.

5) Scope of License; Limitations and Obligations. All Works and all rights therein, including copyright rights, remain the sole and exclusive property of the Rightsholder. The License provides only those rights expressly set forth in the terms and conveys no other rights in any Works

6) General Payment Terms. User may pay at time of checkout by credit card or choose to be invoiced. If the User chooses to be invoiced, the User shall: (i) remit payments in the manner identified on specific invoices, (ii) unless otherwise specifically stated in an Order Confirmation or separate written agreement, Users shall remit payments upon receipt of the relevant invoice from CCC, either by delivery or notification of availability of the invoice via the Marketplace platform, and (iii) if the User does not pay the invoice within 30 days of receipt, the User may incur a service charge of 1.5% per month or the maximum rate allowed by applicable law, whichever is less. While User may exercise the rights in the License immediately upon receiving the Order Confirmation, the License is automatically revoked and is null and void, as if it had never been issued, if CCC does not receive complete payment on a timely basis.

7) General Limits on Use. Unless otherwise provided in the Order Confirmation, any grant of rights to User (i) involves only the rights set forth in the Terms and does not include subsequent or additional uses, (ii) is non-exclusive and non-transferable, and (iii) is subject to any and all limitations and restrictions (such as, but not limited to, limitations on duration of use or circulation) included in the Terms. Upon completion of the licensed use as set forth in the Order Confirmation, User shall either secure a new permission for further use of the Work(s) or immediately cease any new use of the Work(s) and shall render inaccessible (such as by deleting or by removing or severing links or other locators) any further copies of the Work. User may only make alterations to the Work if and as expressly set forth in the Order Confirmation. No Work may be used in any way that is defamatory, violates the rights of third parties (including such third parties' rights of copyright, privacy, publicity, or other tangible or intangible property), or is otherwise illegal, sexually explicit, or obscene. In addition, User may not conjoin a Work with any other material that may result in damage to the reputation of the Rightsholder. User agrees to inform CCC if it becomes aware of any infringement of any rights in a Work and to cooperate with any reasonable request of CCC or the Rightsholder in connection therewith.

8) Third Party Materials. In the event that the material for which a License is sought includes third party materials (such as photographs, illustrations, graphs, inserts and similar materials) that are identified in such material as having been used by permission (or a similar indicator), User is responsible for identifying, and seeking separate licenses (under this Service, if available, or otherwise) for any of such third party materials; without a separate license, User may not use such third party materials via the License.

9) Copyright Notice. Use of proper copyright notice for a Work is required as a condition of any License granted under the Service. Unless otherwise provided in the Order Confirmation, a proper copyright notice will read substantially as follows: "Used with permission of [Rightsholder's name], from [Work's title, author, volume, edition number and year of copyright]; permission conveyed through Copyright Clearance Center, Inc." Such notice must be provided in a reasonably legible font size and must be placed either on a cover page or in another location that any person, upon gaining access to the material which is the subject of a permission, shall see, or in the case of republication Licenses, immediately adjacent to the Work as used (for example, as part of a by-line or footnote) or in the place where substantially all other credits or notices for the new work containing the republished Work are located. Failure to include the required notice results in loss to the Rightsholder and CCC, and the User shall be liable to pay liquidated damages for each such failure equal to twice the use fee specified in the Order Confirmation, in addition to the use fee itself and any other fees and charges specified.

10) Indemnity. User hereby indemnifies and agrees to defend the Rightsholder and CCC, and their respective employees and directors, against all claims, liability, damages, costs, and expenses, including legal fees and expenses, arising out of any use of a Work beyond the scope of the rights granted herein and in the Order Confirmation, or any use of a Work which has been altered in any unauthorized way by User, including claims of defamation or infringement of rights of copyright, publicity, privacy, or other tangible or intangible property.

11) Limitation of Liability. UNDER NO CIRCUMSTANCES WILL CCC OR THE RIGHTSHOLDER BE LIABLE FOR ANY DIRECT, INDIRECT, CONSEQUENTIAL, OR INCIDENTAL DAMAGES (INCLUDING WITHOUT LIMITATION DAMAGES FOR LOSS OF BUSINESS PROFITS OR INFORMATION, OR FOR BUSINESS INTERRUPTION) ARISING OUT OF THE USE OR INABILITY TO USE A WORK, EVEN IF ONE OR BOTH OF THEM HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. In any event, the total liability of the Rightsholder and CCC (including their respective employees and directors) shall not exceed the total amount actually paid by User for the relevant License. User assumes full liability for the actions and omissions of its principals, employees, agents, affiliates, successors, and assigns.

12) Limited Warranties. THE WORK(S) AND RIGHT(S) ARE PROVIDED "AS IS." CCC HAS THE RIGHT TO GRANT TO USER THE RIGHTS GRANTED IN THE ORDER CONFIRMATION DOCUMENT. CCC AND THE RIGHTSHOLDER DISCLAIM ALL OTHER WARRANTIES RELATING TO THE WORK(S) AND RIGHT(S), EITHER EXPRESS OR IMPLIED, INCLUDING WITHOUT LIMITATION IMPLIED WARRANTIES OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE. ADDITIONAL RIGHTS MAY BE REQUIRED TO USE ILLUSTRATIONS, GRAPHS, PHOTOGRAPHS, ABSTRACTS, INSERTS, OR OTHER PORTIONS OF THE WORK (AS OPPOSED TO THE ENTIRE WORK) IN A MANNER CONTEMPLATED BY USER; USER UNDERSTANDS AND AGREES THAT NEITHER CCC NOR THE RIGHTSHOLDER MAY HAVE SUCH ADDITIONAL RIGHTS TO GRANT.

13) Effect of Breach. Any failure by User to pay any amount when due, or any use by User of a Work beyond the scope of the License set forth in the Order Confirmation and/or the Terms, shall be a material breach of such License. Any breach not cured within 10 days of written notice thereof shall result in immediate termination of such License without further notice. Any unauthorized (but licensable) use of a Work that is terminated immediately upon notice thereof may be liquidated by payment of the Rightsholder's ordinary license price therefor; any unauthorized (and unlicensable) use that is not terminated immediately for any reason (including, for example, because materials containing the Work cannot reasonably be recalled) will be subject to all remedies available at law or in equity, but in no event to a payment of less than three times the Rightsholder's ordinary license price for the most closely analogous licensable use plus Rightsholder's and/or CCC's costs and expenses incurred in collecting such payment.

14) Additional Terms for Specific Products and Services. If a User is making one of the uses described in this Section 14, the additional terms and conditions apply:

a) *Print Uses of Academic Course Content and Materials (photocopies for academic coursepacks or classroom handouts).* For photocopies for academic coursepacks or classroom handouts the following additional terms apply:

i) The copies and anthologies created under this License may be made and assembled by faculty members individually or at their request by on-campus bookstores or copy centers, or by off-campus copy shops and other similar entities.

ii) No License granted shall in any way: (i) include any right by User to create a substantively non-identical copy of the Work or to edit or in any other way modify the Work (except by means of deleting material immediately preceding or following the entire portion of the Work copied) (ii) permit "publishing ventures" where any particular anthology would be systematically marketed at multiple institutions.

iii) Subject to any Publisher Terms (and notwithstanding any apparent contradiction in the Order Confirmation arising from data provided by User), any use authorized under the academic pay-per-use service is limited as follows:

A) any License granted shall apply to only one class (bearing a unique identifier as assigned by the institution, and thereby including all sections or other subparts of the class) at one institution;

B) use is limited to not more than 25% of the text of a book or of the items in a published collection of essays, poems or articles;

C) use is limited to no more than the greater of (a) 25% of the text of an issue of a journal or other periodical or (b) two articles from such an issue;

D) no User may sell or distribute any particular anthology, whether photocopied or electronic, at more than one institution of learning;

E) in the case of a photocopy permission, no materials may be entered into electronic memory by User except in order to produce an identical copy of a Work before or during the academic term (or analogous period) as to which any particular permission is granted. In the event that User shall choose to retain materials that are the subject of a photocopy permission in electronic memory for purposes of producing identical copies more than one day after such retention (but still within the scope of any permission granted), User must notify CCC of such fact in the applicable permission request and such retention shall constitute one copy actually sold for purposes of calculating permission fees due; and

F) any permission granted shall expire at the end of the class. No permission granted shall in any way include any right by User to create a substantively non-identical copy of the Work or to edit or in any other way modify the Work (except by means of deleting material immediately preceding or following the entire portion of the Work copied).

iv) Books and Records; Right to Audit. As to each permission granted under the academic pay-per-use Service, User shall maintain for at least four full calendar years books and records sufficient for CCC to determine the numbers of copies made by User under such permission. CCC and any representatives it may designate shall have the right to audit such books and records at any time during User's ordinary business hours, upon two days' prior notice. If any such audit shall determine that User shall have underpaid for, or underreported, any photocopies sold or by three percent (3%) or more, then User shall bear all the costs of any such audit; otherwise, CCC shall bear the costs of any such audit. Any amount determined by such audit to have been underpaid by User shall immediately be paid to CCC by User, together with interest thereon at the rate of 10% per annum from the date such amount was originally due. The provisions of this paragraph shall survive the termination of this License for any reason.

b) *Digital Pay-Per-Uses of Academic Course Content and Materials (e-coursepacks, electronic reserves, learning management systems, academic institution intranets).* For uses in e-coursepacks, posts in electronic reserves, posts in learning management systems, or posts on academic institution intranets, the following additional terms apply:

i) The pay-per-uses subject to this Section 14(b) include:

A) Posting e-reserves, course management systems, e-coursepacks for text-based content, which grants authorizations to import requested material in electronic format, and allows electronic access to this material to members of a designated college or university class, under the direction of an instructor designated by the college or university, accessible only under appropriate electronic controls (e.g., password);

B) Posting e-reserves, course management systems, e-coursepacks for material consisting of photographs or other still images not embedded in text, which grants not only the authorizations described in Section 14(b)(i)(A) above, but also the following authorization: to include the requested material in course materials for use consistent with Section 14(b)(i)(A) above, including any necessary resizing, reformatting or modification of the resolution of such requested material (provided that such modification does not alter the underlying editorial content or meaning of the requested material, and provided that the resulting modified content is used solely within the scope of, and in a manner consistent with, the particular authorization described in the Order Confirmation and the Terms), but not including any other form of manipulation, alteration or editing of the requested material;

C) Posting e-reserves, course management systems, e-coursepacks or other academic distribution for audiovisual content, which grants not only the authorizations described in Section 14(b)(i)(A) above, but also the following authorizations: (i) to include the requested material in course materials for use consistent with Section 14(b)(i)(A) above; (ii) to display and perform the requested material to such members of such class in the physical classroom or remotely by means of streaming media or other video formats; and (iii) to "clip" or reformat the requested material for purposes of time or content management or ease of delivery, provided that such "clipping" or reformatting does not alter the underlying editorial content or meaning of the requested material and that the resulting material is used solely within the scope of, and in a manner consistent with, the particular authorization described in the Order Confirmation and the Terms. Unless expressly set forth in the relevant Order Conformation, the License does not authorize any other form of manipulation, alteration or editing of the requested material.

ii) Unless expressly set forth in the relevant Order Confirmation, no License granted shall in any way: (i) include any right by User to create a substantively non-identical copy of the Work or to edit or in any other way modify the Work (except by means of deleting material immediately preceding or following the entire portion of the Work copied or, in the case of Works subject to Sections 14(b)(1)(B) or (C) above, as described in such Sections) (ii) permit "publishing ventures" where any particular course materials would be systematically marketed at multiple institutions.

iii) Subject to any further limitations determined in the Rightsholder Terms (and notwithstanding any apparent contradiction in the Order Confirmation arising from data provided by User), any use authorized under the electronic course content pay-per-use service is limited as follows:

A) any License granted shall apply to only one class (bearing a unique identifier as assigned by the institution, and thereby including all sections or other subparts of the class) at one institution;

B) use is limited to not more than 25% of the text of a book or of the items in a published collection of essays, poems or articles;

C) use is limited to not more than the greater of (a) 25% of the text of an issue of a journal or other periodical or (b) two articles from such an issue;

D) no User may sell or distribute any particular materials, whether photocopied or electronic, at more than one institution of learning;

E) electronic access to material which is the subject of an electronic-use permission must be limited by means of electronic password, student identification or other control permitting access solely to students and instructors in the class;

F) User must ensure (through use of an electronic cover page or other appropriate means) that any person, upon gaining electronic access to the material, which is the subject of a permission, shall see:

- a proper copyright notice, identifying the Rightsholder in whose name CCC has granted permission,
- a statement to the effect that such copy was made pursuant to permission,
- a statement identifying the class to which the material applies and notifying the reader that the material has been made available electronically solely for use in the class, and
- a statement to the effect that the material may not be further distributed to any person outside the class, whether by copying or by transmission and whether electronically or in paper form, and User must also ensure that such cover page or other means will print out in the event that the person accessing the material chooses to print out the material or any part thereof.

G) any permission granted shall expire at the end of the class and, absent some other form of authorization, User is thereupon required to delete the applicable material from any electronic storage or to block electronic access to the applicable material.

iv) Uses of separate portions of a Work, even if they are to be included in the same course material or the same university or college class, require separate permissions under the electronic course content pay-per-use Service. Unless otherwise provided in the Order Confirmation, any grant of rights to User is limited to use completed no later than the end of the academic term (or analogous period) as to which any particular permission is granted.

v) Books and Records; Right to Audit. As to each permission granted under the electronic course content Service, User shall maintain for at least four full calendar years books and records sufficient for CCC to determine the numbers of copies made by User under such permission. CCC and any representatives it may designate shall have the right to audit such books and records at any time during User's ordinary business hours, upon two days' prior notice. If any such audit shall determine that User shall have underpaid for, or underreported, any electronic copies used by three percent (3%) or more, then User shall bear all the costs of any such audit; otherwise, CCC shall bear the costs of any such audit. Any amount determined by such audit to have been underpaid by User shall immediately be paid to CCC by User, together with interest thereon at the rate of 10% per annum from the date such amount was originally due. The provisions of this paragraph shall survive the termination of this license for any reason.

c) *Pay-Per-Use Permissions for Certain Reproductions (Academic photocopies for library reserves and interlibrary loan reporting) (Non-academic internal/external business uses and commercial document delivery).* The License expressly excludes the uses listed in Section (c)(i)-(v) below (which must be subject to separate license from the applicable Rightsholder) for: academic photocopies for library reserves and interlibrary loan reporting; and non-academic internal/external business uses and commercial document delivery.

i) electronic storage of any reproduction (whether in plain-text, PDF, or any other format) other than on a transitory basis;

ii) the input of Works or reproductions thereof into any computerized database;

iii) reproduction of an entire Work (cover-to-cover copying) except where the Work is a single article;

iv) reproduction for resale to anyone other than a specific customer of User;

v) republication in any different form. Please obtain authorizations for these uses through other CCC services or directly from the rightsholder.

Any license granted is further limited as set forth in any restrictions included in the Order Confirmation and/or in these Terms.

d) *Electronic Reproductions in Online Environments (Non-Academic-email, intranet, internet and extranet).* For "electronic reproductions", which generally includes e-mail use (including instant messaging or other electronic transmission to a defined group of recipients) or posting on an intranet, extranet or Intranet site (including any display or performance incidental thereto), the following additional terms apply:

i) Unless otherwise set forth in the Order Confirmation, the License is limited to use completed within 30 days for any use on the Internet, 60 days for any use on an intranet or extranet and one year for any other use, all as measured from the "republication date" as identified in the Order Confirmation, if any, and otherwise from the date of the Order Confirmation.

ii) User may not make or permit any alterations to the Work, unless expressly set forth in the Order Confirmation (after request by User and approval by Rightsholder); provided, however, that a Work consisting of photographs or other still images not embedded in text may, if necessary, be resized, reformatted or have its resolution modified without additional express permission, and a Work consisting of audiovisual content may, if necessary, be "clipped" or reformatted for purposes of time or content management or ease of delivery (provided that any such resizing, reformatting, resolution modification or "clipping" does not alter the underlying editorial content or meaning of the Work used, and that the resulting material is used solely within the scope of, and in a manner consistent with, the particular License described in the Order Confirmation and the Terms.

15) Miscellaneous.

a) User acknowledges that CCC may, from time to time, make changes or additions to the Service or to the Terms, and that Rightsholder may make changes or additions to the Rightsholder Terms. Such updated Terms will replace the prior terms and conditions in the order workflow and shall be effective as to any subsequent Licenses but shall not apply to Licenses already granted and paid for under a prior set of terms.

b) Use of User-related information collected through the Service is governed by CCC's privacy policy, available online at www.copyright.com/about/privacy-policy/.

c) The License is personal to User. Therefore, User may not assign or transfer to any other person (whether a natural person or an organization of any kind) the License or any rights granted thereunder; provided, however, that, where applicable, User may assign such License in its entirety on written notice to CCC in the event of a transfer of all or substantially all of User's rights in any new material which includes the Work(s) licensed under this Service.

d) No amendment or waiver of any Terms is binding unless set forth in writing and signed by the appropriate parties, including, where applicable, the Rightsholder. The Rightsholder and CCC hereby object to any terms contained in any writing prepared by or on behalf of the User or its principals, employees, agents or affiliates and purporting to govern or otherwise relate to the License described in the Order Confirmation, which terms are in any way inconsistent with any Terms set forth in the Order Confirmation, and/or in CCC's standard operating procedures, whether such writing is prepared prior to, simultaneously with or subsequent to the Order Confirmation, and whether such writing appears on a copy of the Order Confirmation or in a separate instrument.

e) The License described in the Order Confirmation shall be governed by and construed under the law of the State of New York, USA, without regard to the principles thereof of conflicts of law. Any case, controversy, suit, action, or proceeding arising out of, in connection with, or related to such License shall be brought, at CCC's sole discretion, in any federal or state court located in the County of New York, State of New York, USA, or in any federal or state court whose geographical jurisdiction covers the location of the Rightsholder set forth in the Order Confirmation. The parties expressly submit to the personal jurisdiction and venue of each such federal or state court.