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Review Article

Lipopolysaccharide: An indispensable source for potential targets and therapeutic design against Gram-negative bacteria

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Abstract

Gram-negative bacteria show more drug-resistant than Gram-positive bacteria due to unique structural attribute and cause significant morbidity and mortality across the globe. Such characteristic structure is an organelle lipopolysaccharide (LPS) on the outer membrane (OM) of cell wall essential for growth and survival of bacteria. LPS is a major cell wall component formed by dedicated transenvelope multiprotein complexes that shield the underlying peptidoglycan layer and play a key role in host–pathogen interactions with the innate immune system. Moreover, which constitutes the surface-exposed molecules with lipid portion in the outer leaflet of the OM that able to show antibiotic resistance and also responsible for the variety of biological effects associated with bacterial sepsis. LPS synthesis and structure are a conserved subject in infections during bacterial adaptive changes. Such changes ensue immune evasion, prolonged inflammation and augmented antibiotic resistance by working as molecular decoys which titrate the antimicrobials away from its intracellular antibiotic target. Herein, this review summarises the key features of LPS structure, function and biosynthesis. Moreover, it highlights the broad-spectrum conserved targets in the Raetz pathway without an alternative way for LPS biosynthesis vital for the development of novel therapeutic interventions against Gram-negative pathogens.

Keywords: Gram-negative pathogen, lipid A, lipopolysaccharide, O-antigen, potential drug targets

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OVERVIEW

In prokaryotes, eubacteria are typically grouped into two classes such as Gram-negative and Gram-positive bacteria based on their response to a Gram stain. Each group of bacteria has a characteristic spectrum of structural components and fundamental differences within the composition and organisation of their respective cell walls. In Gram-positive bacteria, plasma membranes are encapsulated by a multilayered, crosslinked polymer of peptidoglycan (PG), whereas the PG is further surrounded by a monolayer in Gram-negative bacteria.[1] Regarding the differences in cell envelope structure, these classified bacteria have differential susceptibility to a wide variety of antimicrobial agents. However, Gram-negative

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bacteria are more intrinsically resistant to antibiotics than Gram-positive bacteria; it leads to considerable morbidity and mortality across the globe.

The outer membrane (OM) of the Gram-negative bacteria is a lipid bilayer interspersed with proteins similar to the plasma membrane. The inner leaflet of this membrane is comprised glycerophospholipids including primarily phosphatidylethanolamine, cardiolipin and lesser amounts of phosphatidylglycerol, whereas the lipid of its outer leaflet is composed of amphiphilic glycolipids termed lipopolysaccharide (LPS) molecules. It is a predominantly potent activator of the innate immune system of animals and causes septic shock.[2] The unique structural characteristics are the essential factors for the selective permeability and barrier function of the OM.[1] Specifically, LPS is to form a layer stabilised by divalent cations, and it facilitates an effective permeability barrier against detrimental molecules including antibacterial agents and also antimicrobial peptides.[3] Thus, the LPS became a central component of the OM in Gram-negative bacteria, and moreover, it commonly plays a vital role in pathogenesis in host organisms.[4]

The typical LPS is structurally distinguished with three parts, namely lipid A, core oligosaccharide and O antigen polysaccharide. In LPS, lipid A and core collectively form 'rough portion' by lipooligosaccharides, while 'smooth' portion of the LPS capped with O antigen. In which, lipid A portion is hydrophobic moiety embedded in the OM which helps in attaching LPS to the outer leaflet of the OM.[3]

Lipid A is a glycolipid core of LPS and its lipid component of an endotoxin is responsible for the toxicity of Gram-negative bacteria in disease causing and death from Gram-negative sepsis, an important cause of human mortality and morbidity.[2] It is composed of acyl chains connected to glucosamine backbone by ester and/or amide bonds. Hexa-acylated lipid A induces sturdy inflammatory responses upon recognition by macrophages, monocytes and dendritic cells through the complex toll-like receptor 4 and myeloid differentiation factor 2.[5] Altering the lipid A acylation patterns, or substituting positively charged factors to the lipid A phosphate groups,[6] contributes protection against host innate immunity as well as diminishes the permeability of the OM to antimicrobial peptides and dampening inflammatory responses by the host.[3,7,8]

The second portion is core oligosaccharide associated with lipid A, which maintains the integrity of the OM; the rest of the core comprises a group of sugars which differs amongst species and even amongst strains of the same species.[6] Core sugars phosphorylation is associated with enhanced membrane impermeability and resistance to antibiotics.[9] The core part acts as a mediator and ligand for the transport of LPS to the OM and for the cystic fibrosis (CF) transmembrane conductance regulator protein, respectively.[10] O-antigen polysaccharide or O-antigen is linked to the core and possessed repeating oligosaccharide units in straight interaction with the external locale.[3,4]

O-antigen polysaccharide is found with linear or branched type of oligosaccharide units.[4] O-repeating units are highly variable and involved in the formation of huge number of immunochemically different O-specific serotypes.[11] Moreover, the O-antigen contributes swimming, swarming motility and protection against oxidative stress and also aids in the evasion process from the host immune defenses, especially which evade the complement cascade.[12,13] Structurally and serologically distinct O-antigen molecules were identified such as 'A-band' or 'common polysaccharide antigen', a homopolymer of d-rhamnose. 'B-band' or 'O-specific antigens' another kind of O-antigens which show strong antibody response by immunogenic heteropolymers made up of repetitive units of different sugars.[14,15] The nature of different cell composition including LPS of bacteria naturally leads to disease progression and adaptation to the host milieu and remains persist the lifetime of the patient, for example respiratory infections of CF and gastric infections by *Helicobacter pylori*.[15]

The adverse side effects of anti-bacterial agents vary from regimen to the regimen. Noticeably, despite the drug resistance rates are increasing by fostering the development of more resistant strains.[16] Consequently, a new focus has been required to fight against multidrug resistance bacteria by academics and clinicians with effective studies. Therefore, these potential problems must be reckoned with the triaging of hits require a consideration of chemical tractability for success of novel proof-of concept leads and breakthrough medicines against conserved drug targets.[17] Choosing molecular targets and computer-aided drug designing aspects for new antibiotics seem to be a good basis to overcome these problems. In this perspective, LPS molecule has attained a great attention and became a wide spectrum therapeutic target for the development of novel antimicrobial agents as its involvement in the diverse immunological properties and plays crucial roles in OM stability for cell viability. This review hence summarises parallelly the structural importance of LPS molecule for the bacteria, biosynthesis

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and contribution of the therapeutic targets for drug discovery processes.

LPS-BIOSYNTTHESIS

Lipid A-biosynthesis

The constitutive enzymatic pathway of lipid A biosynthesis present in virtually all Gram-negative bacteria is viewed as a conserved and a variable component as it contains the intracellular and constitutive enzymes.[7] Literature study reported that the LPS biosynthesis was elaborated in detail elsewhere.[3,4,7,14,18] In brief, the lipid A is synthesised on the cytoplasmic side of the inner membrane through a constitutive lipid A pathway. This lipid-A pathway is comprised nine conserved enzymes. Each enzyme is encoded by a single-copy structural gene in Gram-negative bacteria for lipid-A biosynthesis.[19] These nine enzymes synthesise the lipid-A by their sequential catalytic mechanisms with the conversion of the precursor UDP-*N*-acetylglucosamine into lipid A-Kdo2, which is the essential component for the rest of the core sugars which are appended from nucleotide sugar precursors through sequential glycosyl transfer reactions.[3,4,6] It is synthesised on the cytoplasmic side of the inner membrane. Further diverse covalent modifications of lipid-A are occurred while transit from inner membrane to the outer leaflet of the OM.[3,7] Phosphorylation of the core sugars is associated with augmented membrane impermeability and drug resistance in microbes as well as is required for the LPS transport to the OM.[9,10]

The lipid-A genes are sequentially followed: LpxA (UDP-N-acetylglucosamine acyltransferase), LpxC (UDP-3-O-(R-3-hydroxyacyl)-N-acetylglucosamine deacetylase) and LpxD (UDP-3-O-(R-3-hydroxyacyl) glucosamine N-acyltransferase) are cytoplasmic soluble proteins,[20-22] peripheral membrane proteins LpxB (Lipid-A-disaccharide synthase) and LpxH (UDP-2,3-diacylglucosamine-specific pyrophosphatase);[23-25] whereas the distal enzymes of the pathway such as LpxK (Tetraacyldisaccharide 4'-kinase), KdtA (3-deoxy-D-manno-2-octulosonic acid (Kdo) transferase or WaaA), LpxL (Lipid A biosynthesis lauroyltransferase) and LpxM (Lipid A biosynthesis myristoyltransferase) are integral inner membrane proteins.[7,26-29]

In Raetz pathway, non-LpxL-LpxM type enzymes such as LpxL, LpxJ (Kdo2-lipid IVA 3' secondary acyltransferase), LpxE (lipid A 1-phosphatase), EptA (lipid A ethanolaminephosphotransferase), KdoH (3-deoxy-D-manno-octulosonic acid-hydrolase), LpxF (lipid A 4'-phosphatase) and LpxR (lipid A 3-O-deacylase) are involved in KDO-lipid-A (tetraacylated) biosynthesis from KDO_2-lipid IVA. The active sites of these enzymes face the cytoplasmic surface of the inner membrane and their water-soluble co-substrates are cytoplasmic molecules.[7]

Core-oligosaccharide biosynthesis

After Kdo2-lipid-A, the inner core oligosaccharide is extended by sequential addition of sugar residues from activated sugar-nucleotide precursors by specific glycosyltransferases.[1] These enzymes are peripheral membrane proteins associated with the inner leaflet of the plasma membrane. Transfer of the first two Kdo residues of the inner core is catalysed by a single, bifunctional enzyme called WaaA (formerly KdtA). KdsB, KdsA, "waa" gene such as waaP, waaC, waaF, waaA, waaZ, waaY, waaQ, waaG, waaB, waaO, waaR, waaU and waaL of the central operon encode the enzymes catalysing each transfer essential for the core oligosaccharide biosynthesis.[1] The inhibition of any initial enzyme of the core biosynthetic pathway that affects the addition of a main chain sugar and followed by the addition of all subsequent sugars including the O antigen.

O-antigen biosynthesis

Cytoplasmic membrane-associated enzyme complexes synthesise the O antigen separately on a lipid carrier molecule C_{55}-undecaprenyl phosphate (Und-P), which is a C_{55}-polyisoprenoid derivative embedded in the plasma membrane and acts as an acceptor for O antigen chain assembly.[1,11] An initiation step starts with transfer of a sugar phosphate residue onto the lipid carrier (UndP) from a nucleotide-activated donor (precursor) with forming a pyrophosphate linkage, and this lipid carrier is used for subsequent O antigen biosynthesis. Although the huge number of O antigen structures have been characterised, only two types of initiating enzymes were found such as WecA (a UDPGlcNAc: undecaprenyl-phosphate GlcNAc-1-phosphate transferase), common initiating enzyme previously termed as Rfe and its homologues. It transfers a GlcNAc-phosphate from UDP-GlcNAc to UndP and forms UndPP-GlcNAc, serves as an acceptor for the assembly of the O chain backbone, composed of alternating GlcNAc and Gal residues.[30] Then, these linear polysaccharides are supposed to become Lewis antigens by the activity of various fucosyltransferases and then forms lipid-linked glycan.[31] However, the other types of enzymes are WbaP family. Both of these are integral membrane proteins with similar hydropathy profiles.[32] Due to the conservation of the initiating reaction, O antigen biosynthesis offers an opportunity to develop inhibitors towards LPS.[1]

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The lipid-linked glycan is then translocated to the periplasm and also polymerised by the proteins, namely Wzy, Wzz and Wzx acting as polymerase, chain-length regulator and flippase, respectively. Finally, the O polysaccharide is transferred from undecaprenyl pyrophosphate (UndPP) onto the lipid A-core by the O antigen ligase WaaL (O-antigen ligase). The most of the enzymes for O antigen assembly are encoded by cluster of genes at the wb* (previously rfb) locus. These loci are highly polymorphic and so showed high diversity of O antigen structures.[18,19]

Moreover, the synthesised O antigen is then exported by three pathways, namely Wzy-dependent, ABC transporter-dependent and synthase dependent pathway.[3,18] Subsequently, the synthesised and mature LPS molecule is transported across the periplasm and placed into the outer leaflet of the OM by the conserved LPS transport (Lpt) pathway.[30,32-34] Further, several proteins such as LPS export system proteins (LptC and LptA) and inner membrane protein (YhjD), OM proteins LptD-E, YtfN, YfgH and YceK harbour and uphold the transfer of LPS across the periplasm, then responsible for the correct insertion of LPS in the outer leaflet.[3,34,35] The most of all bacteria consist of genes involved in O antigen biosynthesis are clustered in a single locus, which provides horizontal gene transfer and regulation of O antigen synthesis.[19,30]

DRUG TARGETS

Lipid-A drug targets

LpxA is a first enzyme target in the LPS synthesis of Gram-negative bacteria for novel antibacterial drug discovery.[36] LpxA was reported as a potential target in different pathogens such as *Acinetobacter baumannii* and *Moraxella catarrhalis*[37,38] LpxA, LpxC LpxB and LpxD of lipid A synthesis are emerged as an attractive molecular targets.[17,39-41] LpxC was identified as a paramount crucial drug target against several pathogenic bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*;[31] *Aquifex aeolicus,*[42] *Leptospira serovar*[41] and 53 *H. pylori* strains;[33,40] LpxA and LpxD were experimentally confirmed as vital for viability of *P. Aeruginosa*.[43] In latest, lpxB gene in *Acinetobacter baumannii* was reported as a potential therapeutic target.[44] Then, LpxD was reported as an attractive antibacterial target along with the development of experimental inhibitors and peptides to this chemically validated target in *E. coli*.[45,46] Lipid A 1-phosphatase, LpxE and LpxH were also identified as drug targets in in *E. coli*.[47,48] However, many Gram-negative bacteria encode LpxI has no sequence similarity to LpxH but produce the same products by an alternative route, for instance *Caulobacter crescentus*.[49] LpxK as an essential target responsible for Kdo(2)-lipid-A, a conserved substructure of LPS and it plays crucial roles in survival and interaction with host organisms.[47,50] LpxL acyltransferase was determined as a target required for normal growth and penta-acylation of lipid A in *Neisseria meningitides, Pseudomonas putida, Burkholderia cenocepacia* and *K. Pneumonia*.[51-55] Lipid-A acyltransferase LpxM possessed a dual activity mechanism is an important for the pathogenicity and biological activity of pathogenic *E. coli* strain, therefore, which indicate LpxM as a vital target for antimicrobial design.[56] In a nutshell, all these enzyme targets were identified but not all nine enzymes are present in all Gram-negative bacteria and some genes were arisen form gene duplication events. The common targets LpxB, LpxC, LpxD, GmhA, KdtA and RfaE2 (ADP-heptose synthase) were reported for *Leptospira interrogans* serovars Copenhageni and Lai.[41]

Drug targets from core-oligosaccharide

Core-oligosaccharide is essential for the virulence and antibiotic resistance. Moreover, it is highly conserved in Gram-negative bacteria. Thus, it is suitable to target the pathway to explore potential common drug–drug targets against the bacterial species or strains.[33] Target proteins KdsA, KdsB1, GmhA, KdtA and RfaE2 (ADP-heptose synthase) from core region of LPS were also reported for *Leptospira interrogans* serovars Copenhageni and Lai.[41] Interestingly, kdtA gene of *Chlamydia pneumonia* was preferred as a target in detection of human aortic tissue disease.[57] However, kdtA role of *Francisella tularensis* that it is not obviously expressed at the surface, avirulent and elicited partial protection[58] KdsA, KdsB, GmhA, HldE and KdtA were screened as drug targets from the outer and inner core of LPS.[33,41] KdsA, KdsB and GmhA were proposed as common drug targets for *H. pylori, Chlamydophila pneumonia* and *Porphyromonas gingivalis,* these proteins were also separately identified as potential conserved drug targets for 53 *H. pylori* strains.[40,59]

Drug targets from O-antigen

The conservation of this initiating reaction of the O-specific antigen biosynthesis could provide an excellent opportunity as potential drug target to develop inhibitors towards LPS biosynthesis.[1] The wecA and waaL mutant strains of *H. pylori* contain no O-antigens in LPS structure.[30] Moreover, O antigen mutation may adversely affect selected type IV secretion systems, thereby which indicates the significance of the O antigen for the bacterial survival. This target as an important vector while in mutant condition in *P. aeruginosa*.[60] The initiating enzyme (WecA) was reported with important catalytic site residues and a putative reductase DmhB of *Yersinia pseudotuberculosis* as indispensable therapeutic targets

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to inhibit further glycan synthesis.[61,62] Other studies stated that mutant WaaL causes the modification of PG synthesis too and impairs the function of PG layer, which thereby indicating the significance of this enzyme.[63]

The experimental approaches had also been reported the LPS as an indispensable target source for antimicrobial compound discovery apart from screening and identifying drug targets through computational approaches. For instance, LpxC was experimentally allowed to drug design and development processes. As a drug target, LpxC history had begun since early 1980s even before the enzyme discovery and consequently LpxC had become the focus of target-based antibacteriacidal discovery projects more than 20 years in several pharmaceutical and academic research groups. One such the antibacterial discovery research group at Merck Research Laboratories had screened a library of chemical compounds using a galE mutant of *Salmonella* and measured LPS biosynthesis experimentally by monitoring incorporation of radiolabelled galactose into bacteria.[64] Despite intense effort in target identification, yet to focus on potential inhibitor design and approve the novel molecules targeting LPS for therapeutic use because no drug has advanced yet beyond phase I clinical trials. In case of LpxC, eventhough experimentally good drug target, merely, one drug ACHN-975 against LpxC has yet reached human studies, but no drug has advanced yet beyond phase I clinical trials. Later, it was discontinued as well due to local inflammation and toxicity signals found in *in vivo* studies.[64,65] Similarly, a trial for LpxC inhibitor RC-01 was also recently stopped on safety grounds.[66] Moreover, another Phase III clinical trial of the novel *P. aeruginosa*-specific LptD inhibitor murepavadin was terminated due to challenges of unexpected toxicity of a new chemical in acute kidney injury.[67] Some other experimental results which indicated that waaL is a functionally one of the gene clusters for the LPS synthesis as its involvement in the O-antigen side chains to stress adaption and virulence in *E. tarda* as well as reported it as a potential target against this bacterium.[68]

In a capsule, despite LPS being a good target identified even from *in silico* to *in vivo* studies, toxicity of the used chemical substance is becoming a major challenge in potential therapeutics development. Therefore, this article summarises the LPS synthesis and also highlights the drug targets indispensable for drug design, thereby significance of growing body of knowledge, as well as experience which could help to overcome such current hurdles in the translational health research on LPS biology.[64,66]

The most Gram-negative bacteria show the unique characteristic of possessing the LPS in cell wall unlike Gram-positive bacteria. The LPS is the main component of the OM serves as a permeability barrier for many antibiotics. It plays a significant role during host–pathogen interactions and becomes a causative factor of chronic infection. The alterations in the LPS during chronic infection may lead to additional feature of adhesion, colonisation, host immune evasion and adaptation to the host milieu. It comprises three portions – lipid A, the core-oligosaccharide and O antigen synthesised through enzymatic Raetz pathway. LPS-mediated virulence positioned in the endotoxic activity of lipid A, the core and O antigen part to provide resistance against host defense mechanisms. Genetic modifications of LPS lead to the establishment of infection, host immune evasion or lead to the inhibition of the host complement system.[19] Even though, these bacterial species or strains are different, the LPS is found to be common target. Thereby, LPS is clearly an attractive broad-spectrum therapeutic target for the development of novel antimicrobials,[1] however, the unique conserved targets (enzymes) without alternative way to synthesise the same product for the LPS biosynthesis are indispensable. Hence, these common target enzymes from lipid-A, the core and O antigen involved in LPS biosynthesis could open the door to various new avenues for antimicrobial therapy with novel chemical probes against multidrug resistance superbugs.

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