Lipid A as a Drug Target and Therapeutic Molecule

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Abstract

In this review, lipid A, from its discovery to recent findings, is presented as a drug target and therapeutic molecule. First, the biosynthetic pathway for lipid A, the Raetz pathway, serves as a good drug target for antibiotic development. Several assay methods used to screen for inhibitors of lipid A synthesis will be presented, and some of the promising lead compounds will be described. Second, utilization of lipid A biosynthetic pathways by various bacterial species can generate modified lipid A molecules with therapeutic value.

Key Words: Lipid A, Endotoxin, Drug target, Antibacterial, Adjuvant

INTRODUCTION

Lipids have not been a very popular research topic for some time, because their study imposes more technical challenges than other biological molecules, such as proteins, nucleic acids, and carbohydrates. While a lot of questions remain to be answered, it is true that remarkable advances have been recently achieved in the lipid field. In this review, lipid A, from its discovery to recent findings, is presented as a drug target with therapeutic value.

Discovery of lipid A

Lipid A is present mainly in Gram-negative bacteria; however, a recent study indicates it might exist in plants. It functions as a hydrophobic anchor for lipopolysaccharide (LPS) attached to the outer membrane, and it appears to be essential for the growth of cells and in LPS-free mutants. The term lipid A was first coined by Westphal and Luderitz as the lipid rich hydrolytic fragment of LPS, and lipid B was defined as the other, more readily separated portion of phospholipids in Escherichia coli (E. coli) (Brade, 1999). The molecular identities of both lipid species are now well known, and lipid B was defined as the other, more readily separated portion of phospholipids in Escherichia coli (E. coli) (Brade, 1999). The molecular identities of both lipid species are now well known, and lipid B was defined as the other, more readily separated portion of phosphatidylethanolamine. When lipid A was first separated from the bacterial cell envelope, neither its exact molecular identity nor its biosynthetic pathway were known. In the late 1970s, when the structure of lipid A was not yet known, lipid X, a biosynthetic intermediate of lipid A, was identified from an E. coli mutant lacking phosphatidylglycerol (Nishijima and Raetz, 1979). While it was determined that lipid X contains acylated glucosamine (GlcN) and a phosphoryl group, the exact structure had yet to be defined. With the structure of lipid A not available, lipid X could not be shown to be related to lipid A. In 1983, the exact structure of lipid A was determined with the help of mass spectrometry and NMR spectroscopy (Imoto et al., 1983; Strain et al., 1983). Then, the structure of lipid X was revisited, and it was determined that lipid X is an intermediate in lipid A biosynthesis (Takahama et al., 1983). The starting compounds of lipid A biosynthesis are N-acetylglucosamine linked to uridine diphosphate (UDP-GlcNAc), and the acyl chain linked to acyl carrier protein (acyl-ACP). After acylation of UDP-GlcNAc mediated by UDP-GlcNAc acyltransferase or LpxA (Crowell et al., 1986), eight reactions ensue to synthesize Kdo₂-lipid A (Raetz and Whitfield, 2002; Raetz et al., 2007). This pathway is called the Raetz pathway (Fig. 1) as explained below.

Function of lipid A in Gram-negative bacteria

Obviously, the function of lipid A in Gram-negative bacteria is to anchor LPSs to the outer membrane of the cell. Generally, lipid A is considered essential for survival. Some laboratory-grown bacterial strains lack LPS, but not lipid A. The outer membrane of Francisella novicida (F. novicida) contains a lot of lipid A not linked to LPS (Wang et al., 2004). However, there are some results reporting the absence of lipid A in viable Gram-negative bacteria. Moffatt and colleagues reported a mutant of Acinetobacter baumannii in which lipid A biosyn-
thesis is absent. These mutants were isolated from colistin-containing media used to induce colistin resistance. All the isolated mutants showed mutations in the first two enzymes of the Raetz pathway, and LPS was not present (Moffatt et al., 2011). In the case of *Neisseria meningitidis*, cells can grow slowly without lipid A (Steeghs et al., 1998). With the exception of the above, lipid A appears to be essential for the survival of Gram-negative bacteria. In laboratory experiments, inhibition of lipid A in a LPS-deficient mutant results in the inhibition of growth, implying that lipid A functions as more than a lipid anchor for LPS. Other biological roles of lipid A and the identities of its biosynthetic intermediates are still unclear.

**Lipid A as an endotoxin**

As picomolar levels of lipid A are sufficient to induce inflammation in the mammalian immune system by triggering TLR4/MD activation (Beutler and Cerami, 1988), lipid A itself can be considered an endotoxin. We can separate the immunogenicity of LPS from its toxicity; its immunogenicity depends on polysaccharide components and its toxicity is due to the lipid A moiety. While inflammation is necessary to clear up infection, overreaction of the immune system can be fatal, and Gram-negative septic shock can occur. The structure of the LPS bound to the TLR4/MD2 complex (Fig. 2) explains the formation of receptor multimers upon binding of LPS (Park et al., 2009; Ohto et al., 2012). TLR4 and MD2 exist as dimers (TLR4/MD2), and LPS binding to MD2 results in the formation of the TLR4/MD2/LPS complex (2:2:2 complex). The lipid chains in LPS are located in the hydrophobic pocket of MD2, and two phosphate groups in lipid A engage in favorable in-

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**Fig. 1.** The Raetz pathway. The biosynthetic pathway for Kdo₂-lipid A (*E. coli*) is shown. The pathway is redrawn from (Raetz et al., 2007) for simplicity, and to indicate the location of each reaction.
interactions with positively-charged residues in TLR4 and MD2, inducing complex formation. In human cells, the presence of two phosphate and acyloxyacyl groups is necessary to activate fully TLR4-MD-2 (Rietschel et al., 1994). Species-specificities exist among lipid A and its analogs. For example, lipid IV\textsubscript{A}, a tetra-acylated precursor of lipid A, is a weak agonist of TLR-4-MD-2 activity in mouse, while it shows antagonistic activity in human (Montminy et al., 2006). It may be possible to utilize the different lipid A analogs as therapeutics molecules. This will be discussed later.

**BIOSYNTHETIC PATHWAY OF LIPID A AS A DRUG TARGET**

The raetz pathway

As described above, UDP-GlcNAc and acyl-ACP, in the case of E. coli, are substrates for the first step of lipid A biosynthesis. LpxA, the first enzyme in the Raetz pathway, is an acyltransferase mediating the acylation of UDP-GlcNAc and yielding UDP-3-(O)-acyl-GlcNAc. This reaction occurs in the cytoplasm, and is followed by the deacetylation of the GlcNAc moiety, mediated by lpxC (Jackman et al., 1999). This reaction yields UDP-3-(O)-acyl-GlcN as a product. As the equilibrium in the LpxA reaction is toward the substrate, the LpxC reaction is the actual step of commitment to the pathway. Once UDP-3-(O)-acyl-GlcN is formed, another acylation reaction occurs, mediated by LpxD (Vuorio and Vaara, 1995), to yield UDP-2,3-(O)-diacyl-GlcN. These three reactions occur in the cytoplasm, and the enzymes responsible are soluble proteins.

One molecule of UDP-2,3-(O)-diacyl-GlcN, and one molecule of lipid X, formed by the removal of UMP moiety from UDP-2,3-(O)-diacyl-GlcN, undergo lipid A disaccharide formation. The formation of lipid X is mediated by either lpxH (Babinski et al., 2002a; Babinski et al., 2002b) or lpxI (Metzger and Raetz, 2010), depending on the bacterial species, and lipid A disaccharide formation is mediated by lpxB (Crowell et al., 1986). These three enzymes are peripheral membrane proteins, and it appears that the reactions occur in the vicinity of the membrane.

Lipid A disaccharide undergoes further modifications. First, the phosphorylation of lipid A disaccharide results in the formation of lipid IV\textsubscript{a}, a tetra-acylated lipid A species. This reaction is mediated by LpxK (Garrett et al., 1997), a phosphorylase, or a kinase. Second, two Kdo residues are attached to lipid IV\textsubscript{a}, yielding Kdo\textsubscript{2}-IV\textsubscript{A}, and the reaction is mediated by KdtA (White et al., 1997), a Kdo transferase. The second and third reactions, mediated by LpxL (Carty et al., 1999) and LpxM (Clementz et al., 1997), are acylation reactions. Tetra-acylated Kdo\textsubscript{2}-IV\textsubscript{A} is transformed into Kdo\textsubscript{2}-lipid A, the final product in the Raetz pathway. LpxL transfers lauroyl residues while LpxM transfers a myristoyl residue to Kdo\textsubscript{2}-IV\textsubscript{a}. Two reactions occur in a sequential manner: the LpxL reaction occurs before the LpxM reaction, and the LpxM reaction does not occur in the lpxL mutant (Vorachek-Warren et al., 2002). The three enzymes that modify lipid A disaccharide are integral membrane proteins, and the reactions probably occur on the inner surface of the inner membrane.

![Diagram of TLR4/MD2 complex formation](image-url)
In the above paragraphs, nine constitutive enzyme reactions in the Raetz pathway were described succinctly, using mainly *E. coli* as a model system. However, variations exist between bacterial species. First, lpxAs from different bacterial species show different selectivities for acyl chains and sugars. Acyl chain length can be different, either short (C10, *Pseudomonas aeruginosa*; *P. aeruginosa* (Dotson et al., 1998; Wyckoff et al., 1998), or long (*Helicobacter pylori* (Lee and Suh, 2003); C18, *F. novicida* (Wang et al., 2006b)), and the lack of the 3-hydroxy group makes a difference, as we observe myristoyl, not the 3-hydroxymyristoyl group, in the *Chlamydia trachomatis* lpxA reaction (Sweet et al., 2001). Sugar selectivity is observed in the case of *Leptospira interrogans* lpxA, as an amino sugar, UDP-2-acetamido-3-amino-2,3-dideoxy-α-D-glucopyranose, is used, resulting in the formation of amide bonds between the acyl chain and the sugar (Robins et al., 2009). Second, the formation of lipid X from UDP-diacyl-GlcN is mediated by either lpxH or lpxl, depending on the bacterial species. LpxH exists in about two-thirds of Gram-negative bacteria, while lpxl is found in the other one-third (Metzger and Raetz, 2010). LpxH and lpxl are unrelated to each other in their DNA sequences, and the enzyme mechanisms are different. These differences are important considerations in the development of inhibitors against lipid A biosynthesis.

### Lipid A biosynthesis inhibitors

The biosynthetic pathway for lipid A can serve as good drug target for antibiotic development, as lipid A is essential in most Gram-negative bacteria. There have been attempts to screen for inhibitors of lipid A biosynthetic enzymes. One way is to measure the incorporation of radioactive galactose into LPS (Austin et al., 1990) to screen for an inhibitor of LPS biosynthesis. One compound, L-573,655 (Table 1), was selected during a screen of compounds by a research group at Merck Research Laboratories (Onishi et al., 1996). After selection, all nine enzymes of lipid A biosynthesis were assayed individually for sensitivity to inhibition by L-573,655, which turned out to inhibit lpxC, the second enzyme of the Raetz pathway. The inhibition constant for L-573,655 was determined to be 24 μM, and 200 analogs were synthesized to bring the inhibition constant up to ~50 nM by L-161,240 (Table 1). While L-161,240 was an inhibitor of *E. coli* lpxC, the inhibition was not universal: L-161,240 could not inhibit lpxC from other species, such as *P. aeruginosa* or *Aquifex aeolicus* (*A. aeolicus*). Later on, hydroxamate-containing inhibitors, including TU-514 (Table 1), were developed, which turned out to be effective against both *E. coli* and *A. aeolicus* enzymes (Jackman et al., 2000). More hydroxamate-containing compounds were developed yielding several inhibitors, including CHIR-090 (Table 1) with antibacterial activity comparable to that of ciprofloxacin. CHIR-090 and other potent inhibitors have threonyl-hydroxamate in their head groups (McClerren et al., 2005). Based on knowledge of the interactions between LpxC and threonyl-hydroxamate-containing inhibitor, interactions of binding pocket and inhibitor were further increased by attaching bulky groups to threonyl-hydroxamate, as seen in the case of LPC-051 (Liang et al., 2013).

As stated above, the first three enzymes of the Raetz pathway are soluble proteins, while others are non-soluble. The non-soluble ones are either peripheral membrane proteins or integral membrane proteins associated with the membrane. The development of enzyme assay systems is challenging for non-soluble proteins. Even for soluble proteins, the enzyme reactions in the Raetz pathway are hard to reconstitute in test tubes. Assays usually involve the use of radioactive isotope-labeled substrates to measure the enzyme activities, and most of the materials are not commercially available. For example, the lpxA enzyme assay requires lpxA, acylated ACPs, and radio-labeled UDP-GlcNAc, but none of these are commercially available. Therefore enzyme activity assays have not been

#### Table 1. Structures of selected hydroxamic acids with LpxC inhibitory activity

| Compound | Structure | Ki value against *E. coli* LpxC | Reference |
|----------|-----------|-------------------------------|-----------|
| L-573,655 | ![Structure](structure1.png) | 24 μM | Onishi et al., 1996 |
| L-161,240 | ![Structure](structure2.png) | ~50 nM | Onishi et al., 1996 |
| TU-514 | ![Structure](structure3.png) | ~650 nM | Jackman et al., 2000 |
| CHIR-090 | ![Structure](structure4.png) | ~2 nM | McClerren et al., 2005 |
| LPC-051 | ![Structure](structure5.png) | ~0.024 nM | Liang et al., 2013 |
used in high-throughput screening for inhibitors against lipid A biosynthesis. When Pirrung and colleagues developed lpxC inhibitors, they used a disk diffusion assay to test 70 oxazoline hydroxamate compounds (Pirrung et al., 2003).

Recently, a fluorescence based enzyme assay was developed for the lpxA reaction. This assay is based on the formation of free thiol from ACPs (Jenkins and Dotson, 2012): free thiols are exposed after the acyl group is transferred from acyl-ACP to UDP-GlcNAc, and this can be monitored by linking thiol exposition to other enzymatic reactions. While a previous method used a discontinuous radioassay based on thin layer chromatography (Galloway and Raetz, 1990), the new assay is a continuous assay that monitors multiple enzyme reactions on a single plate. The new method would allow higher throughput screening of large compound libraries. While most inhibitors of lipid A biosynthesis target lpxC, it does not mean lpxC is the only target. It was shown, using a phage display screening assay, that lpxA is a good antibacterial target (Benson et al., 2003), and all the enzymes in the Raetz pathway are potential targets for antibiotic agents against Gram-negative bacteria.

**LIPID A ANALOGS WITH THERAPEUTIC VALUE**

Lipid A itself is an endotoxin, and thus has no therapeutic value. However, biosynthetic intermediates and modified lipid A could have clinical applications. Lipid IV<sub>A</sub> is an antagonist of the TLR4 receptor in human cells, while it is an agonist in murine cells. It was initially hoped that lipid IV<sub>A</sub> might be useful as a therapeutic agent for the treatment of Gram-negative septicemia (Golenbock et al., 1991). However, because of the lack of stability of lipid IV<sub>A</sub> upon storage, the development of synthetic analogs was pursued instead, among which E5531 and E5564 were the most notable outcomes (Christ et al., 1995) (Fig. 3). While it is less encouraging that a recent clinical trial of E5564 (eritoran) did not reduce the mortality of septic patients (Opal et al., 2013), there are other possible clinical uses for this compound. For example, E5564 protected mice from lethal influenza infection (Shirey et al., 2013), presenting Toll-like receptors as a new drug target for antiviral therapy (Patel et al., 2014). Lipid X was once considered as an antagonist (Danner et al., 1987); however, later it was shown not to have affinity for the TLR4 receptor (Pohlan et al., 1988).

In the above paragraph, synthetic analogs of lipid A were shown to have therapeutic value. It is also possible to prepare lipid A analogs by combining different biosynthetic enzymes from different bacterial species. As seen in the Kdo<sub>2</sub>-lipid A molecule from *E. coli* (Fig. 1), there are two phosphate groups, and the existence of these two phosphate groups is very important for TLR4/MD2 activation (Rietschel et al., 1994). While lipid A itself is too toxic when there are two phosphate groups present, monophospho-lipid A (MPLA) (Persing et al., 2002) can be prepared and used as an adjuvant, as MPLA partially activates TLR4/MD2. Interestingly, there are some bacterial species, such as *Francisella tularensis*, which produce lipid A devoid of phosphate groups. These species contain extra enzymes, including LpxE (Wang et al., 2004) and LpxF (Wang et al., 2006a), that function as phosphatases to get rid of the phosphate groups in lipid A. The LpxE encoding gene was inserted into a *Salmonella* strain to make MPLA-producing bacteria, in the hope of developing live oral vaccines (Kong et al., 2011; Wang et al., 2013). These bacterial strains could be used to deliver the antigen of choice, and to elicit moderate immune responses via MPLA, which would function as both an antigen presenter and an adjuvant.

It should also be possible to utilize biosynthetic enzymes from different species to prepare lipid A analogs *in vitro*. For example, Pagl. (Trent et al., 2001) and LpxR (Reynolds et al., 2006) exist in *Salmonella* species and function as lipases. These enzymes function in *E. coli*, and modified lipid A species are produced by cultured bacterial cells (Gibbons et al., 2000; Reynolds et al., 2006). It is expected that the repertoire of lipid A modifying enzymes from various bacterial species will allow the production of lipid A analogs with beneficial activities.

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