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Review Article

Novel therapeutic agents against endotoxemia and septic shock

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CONTENTS

Abstract

Upon bacterial infection, the release of lipopolysaccharide (LPS) endotoxin from Gram-negative bacterial membranes triggers the production of large quantities of cytokines in the body, which can lead to septic shock and eventual death. Various therapeutic strategies have been used to target different stages of the condition, *e.g.*, antibodies against LPS or cytokines, with little success in the clinic. Recently, two approaches have provided hope – the use of anticoagulants and cationic peptide-based agents that bind to and neutralize LPS. Here, we focus the review on recent advances towards the development of therapeutic agents against endotoxemia and septic shock.

Introduction

Sepsis is the tenth most common cause of death (greater than from AIDS, breast cancer and heart attacks) (1), and the primary cause of death in intensive care units, with about 200,000 deaths per year in the U.S. alone out of a population of 750,000 patients (2). This translates to a mortality rate in the U.S. of 29%, and worldwide rates of sepsis are around 45%, having increased as a result of greater use of invasive procedures, immunosuppression and chemotherapy. Intensive research into the pathophysiology of sepsis and related areas has been under way for some time, and numerous clinical studies using various therapeutic approaches have been performed through the 1990s to the present time. Even with such intensive investigations of more than 30 therapeutic agents, a truly effective treatment for sepsis has been elusive.

Sepsis is triggered by lipopolysaccharide (LPS) endotoxins in the bloodstream. Gram-negative bacterial infection generally results in the release of these LPS endotoxins from their ruptured membranes (3, 4). Increased levels of LPS in the bloodstream induce a biological response whereby cytokine levels rise to a toxic concentration that generally leads to the systemic complications of sepsis and septic shock. Initially, LPS exposure triggers an inflammatory response that involves various cell receptors (5), for example CD14 (6), the Toll-like receptor 4 (TLR4)-MD-2 receptor complex (7) and non-CD14-expressing endothelial cells (8), and plasma components such as cytokines, lipid mediators and reactive oxygen species (ROS) (9), and this ultimately leads to the cascade of septic shock, organ failure, and ultimately death (10). In the past, acceptable clinical therapy was aimed at combating the bacterial infection with various antibacterial agents, but these agents in themselves lead to disruption of the Gram-negative bacterial membranes that release even more LPS.

Recent clinical strategies against sepsis have been focused on targeting the mediators of sepsis, primarily cytokines. However, this approach has failed in clinical trials (11). A therapeutic approach that suppresses LPS stimulation of the inflammatory response at the onset, rather than one that inhibits any individual intermediate mediator or molecular event, has been attempted with little success, but may actually be the most effective way to halt the septic shock cascade. In this regard, a therapeutic agent that can bind to and neutralize LPS directly would be highly useful in the clinic and highly effective LPS-neutralizing agents are clearly needed.

LPS, which is an integral component of the outer membrane of Gram-negative bacteria (12, 13), is composed of several hydrophobic acyl chains at one end and hydrophilic anion-conjugated carbohydrate groups at the

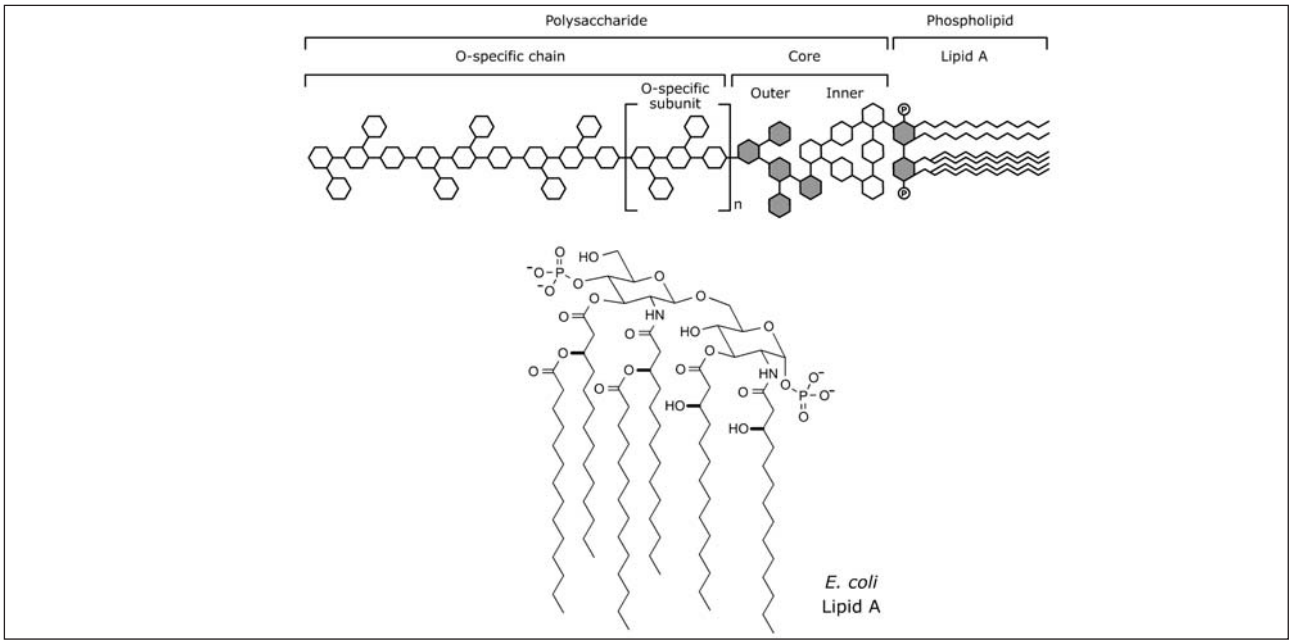

Fig. 1. Illustration of an idealized molecule of lipopolysaccharide (LPS) and lipid A from *Escherichia coli*.

other. The illustration in Figure 1 is a general picture of LPS, because the chemical structure of the molecule is quite variable among different species of bacteria (12, 14). From any Gram-negative species of bacteria, the lipid A group is the most conserved part of LPS. Basically, the lipid A moiety is a poly-*N*- and-*O*-acylated, 4-phosphoglucos-2-amine-[1,6-*O*]-1-phosphoglucos-2-amine. Lipid A is illustrated in Figure 1. Attached to the O6 of one of the glucosamines is a group of phosphorylated polysaccharides (the inner core) and then an outer core of simpler polysaccharides and an *O*-specific chain. These latter chemical elements vary considerably from one Gram-negative bacterium to another. The variability of any of these groups among different molecules of LPS from different bacteria is the primary obstacle to identifying a single broad-spectrum agent that neutralizes LPS equally well from multiple species of bacteria. In this regard, the more conserved lipid A moiety is perhaps the best target against which to design pharmaceutical drugs for therapeutic intervention.

This review focuses on therapeutic agents against sepsis and ongoing research into the development of agents against sepsis. In particular, efforts are under way to create more potent and selective LPS-neutralizing compounds. Designing these novel compounds has been assisted by understanding their mechanism of action and how they interact with LPS.

Approaches to combat sepsis

Early attempts

The late 1990s witnessed a number of clinical trials using various approaches to combat sepsis in patients. However, these had only very limited, if any, success. Because LPS binding to macrophages induces the release of numerous cytokines that lead into the sepsis cascade, agents that act to reduce cytokine levels in septic patients have been tried: IgG-soluble cytokine receptor fusion proteins such as sTNFRp55-IgG (15) and sTNFRp75-IgG (16), the IL-1Ra cytokine receptor antagonist (17) and various anti-cytokine antibodies such as an anti-TNF-α antibody (18). Even though 3-day mortality was slightly improved, there was really no significant benefit in terms of 28-day survival in these trials.

The release of LPS from the bacterial membrane triggers sepsis, and because the lipid A moiety of LPS is most conserved among Gram-negative bacteria, anti-lipid A antibody (HA-1A and -E5) therapies have also been attempted, again with no real success (19, 20). Targeting upstream LPS has also been tried using LPS-binding proteins, *e.g.*, bactericidal/permeability-increasing (BPI) protein (21, 22) and the cathelicidin-derived peptide CAP18 (23, 24) and perfusion with polymyxin B (25, 26). The limited success and/or failure of any of these early or upstream strategies is, in part, probably the result of relatively late treatment of patients with sepsis, given that the sepsis cascade had already been initiated by bacterial infection.

Other approaches have been attempted to disrupt various points in the sepsis cascade: platelet-activating factor (PAF) antagonists (27), anti-prostaglandin therapy (28), bradykinin antagonists (29, 30) and liposomal prostaglandin E_1 (31). Although phase II trials with bradykinin showed initial promise, real clinical benefit has not been established (29).

Hope on the horizon with coagulation inhibitors?

The physiological reaction to bacterial infection can be viewed as a balance between pro- and antiinflamma-

tory responses. Weak proinflammatory and strong antiinflammatory effects may promote infection and lead to death, whereas a strong proinflammatory effect (*i.e.*, release of proinflammatory mediators) may promote microvascular thrombosis and organ failure. As mentioned above, most trials targeting proinflammatory mediators have failed, possibly because this balance among various inflammatory mediators was not thoroughly considered. However, the use of anticoagulant agents, including tissue factor pathway inhibitor (TFPI), antithrombin III (ATIII) and protein C, offers hope (32). Although trials employing TFPI and ATIII were disappointing (33), a 1,690-patient clinical trial using protein C to combat severe sepsis was considered successful, as evidenced by a significant decrease in mortality compared to the placebo group (34). Although considered by some to be a major clinical advance, treatment with protein C only reduced overall mortality by about 6%, from 30.8% in the placebo group to 24.7% in the treatment group ($p = 0.005$) (34), and could be potentially detrimental to patients with severe sepsis (35). However, in a subgroup of patients who had an Acute Physiology and Chronic Health Evaluation (APACHE) (36), mortality was reduced by about 13% (34).

Clearly, stand-alone therapy is insufficient to completely combat the complex condition of sepsis. Current therapy for sepsis is therefore multimodal and is initiated as soon as possible at the recognized onset of sepsis (37). In a recent clinical trial that combined the use of early hemodynamic optimization (EGDT; basically oxygen delivery therapy), glucose control, low-dose glucocorticoids to help relieve acute stress and activated protein C, 30 intensive care unit patients received this therapy and 30 control patients did not. The treated group demonstrated a 26% reduction in mortality compared to the control group (27% *vs.* 53%; $p < 0.05$) (38). Although this clinical result is impressive, there is room for improvement, and research in the field continues.

A renaissance in LPS neutralization with cationic peptides

"Hope runs eternal", as researchers have not given up on attempts to neutralize LPS. Targeting the initiator of septic shock and sepsis has undergone a renaissance or rebirth. The observation stimulating this renaissance is that many cationic peptides, long known for their ability to kill bacteria by disintegrating the bacterial membrane, can also bind to and thereby neutralize LPS (39). Cationic peptides are natural to plants (40), insects (41, 42), amphibians (43) and mammals (44-46). As a type of immune response, injury in insects and amphibians, for example, promotes the release of cationic peptides to prevent local infection. High levels of cationic peptides are also found in neutrophils of mammals to promote a rapid response against microbes.

Presently, over 1,000 cationic peptides are known (47, 48). These peptides vary in length and in their folded conformation, *i.e.*, β-sheet, α-helix, extended structure and covalent or disulfide-looped structure, but regardless of conformation, they all possess two common features: a net positive charge of generally +2 to +6, and an amphipathic nature to their folded structure with polar and hydrophobic faces (49). Because the surface of bacterial membranes is negatively charged, cationic peptides (and related agents) interact and become integrated into the membrane (50), making it leaky and promoting cell lysis (51). This is particularly true of Gram-negative bacteria, which contain the highly negatively charged LPS molecule that is integral to the bacterial membrane. Cationic peptides that form α-helices are exemplified by melittin (52) and magainin (53), as well as by peptaibols (54-56) and lipopeptaibols (57) that contain Cαα-disubstituted α-aminoisobutyric acids as their positively charged groups, an *N*-terminal acetyl or fatty acid group and a *C*-terminal alcohol. Others, like the defensins (58) and the protegrins (59), are amphipathic by virtue of their usual disulfide bond-stabilized β-sheet conformation. Polymyxin B, a well-known and widely used topical antibiotic (60), is also a cationic peptide with a looped structure having several positively charged diaminobutyric acid residues. Other well-studied examples of naturally occurring cationic peptides are the BPI protein (61), the cecropins (62), proline-arginine-rich peptides (63), sapecin (64) and tachyplesin (65). Hybrids of these peptides have also been made in an attempt to amplify the activity of the parent peptides, *e.g.*, a cecropin-melittin hybrid (66) and a cecropin-magainin hybrid (67, 68). Other cationic peptides, such as βpep peptides (69, 70), the dodecapeptide SC4 (71) and a repeating KIGAKI hexapeptide (72), have been designed based on the general amphipathic and cationic nature of these naturally occurring peptides. Many cationic peptides are active against bacteria in the micromolar to submicromolar range, and a few are selective for bacterial cells and do not greatly affect eukaryotic cells at therapeutically effective doses.

Although there is no strong correlation between LPS neutralization and bactericidal effects for cationic peptides, there is a general trend for a bactericidal cationic agent to also, to some extent, bind to and neutralize LPS (39). This is related, or is at least thought to be related, to the bactericidal mechanism of action of these cationic peptides and related compounds. The external leaflet of bacterial cell membranes is composed of zwitterionic phosphatidylethanolamine and anionic phosphatidylglycerol lipids, lipopolysaccharides for Gram-negative bacteria and teichoic acid groups for Gram-positive bacteria, endowing all bacterial membranes a net negative charge, which differentiates them from eukaryotic membranes that exhibit a much reduced negative charge. Cationic charges on the antibacterial peptide promote interaction with the negatively charged bacterial polysaccharides and membranes. In general, the presence of LPS in bacterial membranes of Gram-negative bacteria makes cationic peptides more effective against Gram-negative bacteria compared to Gram-positive bacteria. In fact, truncation of the LPS outer core greatly reduces the susceptibility of bacteria to antimicrobial cationic peptides (73).

LPS neutralization studies have been carried out on many cationic peptides, *e.g.*, the cathelicidin peptides LL-37 (74), CAP37 (75) and CAP11 (76, 77), PR-39 (78), LALF-derived cyclic peptides (79), lactoferrin-based peptides (80), NK-2 (81), histidine-rich peptides (82), the proline-rich peptide Bac7 (83), arginine-rich peptides (84), a tachyplesin I-derived cyclic peptide (85), βpep peptides (69, 70) and the dodecapeptide SC4 (71). A few of these peptides exhibit IC_{50} values against LPS from specific bacterial species in the nanomolar range.

Structural analysis of cationic peptides can be quite helpful in identifying those molecular features paramount to binding to and neutralizing LPS. Regardless of the molecular structure of a given peptide, the underlying requirement for efficient interaction with LPS is known from many studies to be a net positive charge and high hydrophobicity, usually LPS binding in the context of an amphipathic structure. Even the larger and highly effective BPI protein (61) forms an amphipathic β-sheet structural motif having a highly cationic face. Positively charged residues from the peptide/protein presumably promote interaction with negatively charged groups on LPS, *i.e.*, phosphates on the lipid A glucosamines and/or those in the inner core polysaccharide unit, while hydrophobic residues from the peptide interact with acyl chains on lipid A (see Fig. 1).

In this regard, structure-based modifications to a number of these peptides have been made to improve LPS binding affinity via replacement with more hydrophobic and cationic amino acid residues, *e.g.*, CAP18/LL-37 (86, 87). In addition, analogous to polymyxin B, Muhle and co-workers designed a family of cyclic β-sheet peptides composed of putative LPS-binding sequences (88). Other peptides have been designed, *e.g.*, the lactoferrin-based peptide LF11 (89) and the *N*-acylated form lauryl-LF11 (90).

Structural aspects of how cationic peptides interact with LPS

Not all peptides or agents that are cationic (and highly hydrophobic) effectively neutralize LPS. However, despite the diversity of sequences and folded structures of cationic peptides that are effective against LPS, their mechanism of action appears to be similar, and there are some structural features of cationic agents that appear to be necessary for effective binding to LPS. Overall, cationic peptides appear to target the lipid A moiety of LPS (91), and modifications to the lipid A moiety of LPS have been shown to increase or decrease the affinity of cationic peptides for LPS (92). Although one of the advantages of using cationic peptides is their ability to avoid the problem of bacterial resistance as observed with conventional antibiotics, Gram-negative bacteria in some cases can modify their LPS to become resistant to cationic peptides (93). For example, bacterial acylation of lipid A can contribute to the sensitivity to cationic peptides (94), and even the packing of the LPS on the bacterial membrane surface can reduce the ability of these peptides to bind LPS (95). Due to the complexity and compositional heterogeneity of LPS, no structural studies have been performed with LPS, and here we will explore what is structurally known about how cationic agents interact with the lipid A moiety of LPS.

In an X-ray crystal structure of the *Escherichia coli* iron uptake receptor protein FhuA in complex with an LPS molecule, Ferguson *et al.* (96) found that a precise spatial arrangement of cationic side-chains from a three-stranded antiparallel β-sheet was crucial to bind to the lipid A moiety of LPS. Using NMR spectroscopy, Pristovsek and Kidric (97) determined the structure of PmxB in an LPS-bound state and concluded that a phenylalanine (F6) side-chain and two positively charged α,γ-diaminobutyric acid groups (Dab 1 and Dab 5) were crucial for binding to LPS. In this structure of the complex, the polar surface of PmxB falls on top of the glycosidic hydrophilic groups of lipid A, while the hydrophobic chain of PmxB interacts with the acyl chains of lipid A.

Structural studies on peptide LF11 in complex with an LPS molecule from *E. coli* serotype 055:B5 demonstrated that the peptide folded "in a 'T-shaped' arrangement of a hydrophobic core and two clusters of basic residues that match the distance between the two phosphate groups of the lipid A moiety" (89). In this case, two of the arginines from peptide LF11 are positioned close to the two phosphate groups of the lipid A moiety. The distance of about 13 Å separating these two phosphate groups nearly matches that between the two guanidinium groups in the complex. Nevertheless, other arginine and lysine groups in peptide LF11 could also interact with the lipid A phosphate groups. Moreover, these authors mentioned that one of the arginine guanidinium groups may hydrogen bond to one of the sugar units in LPS. The guanidinium group, rather than the ammonium group, forms an inherently stronger electrostatic interaction with the phosphate group compared to the primary amine in lysines (98). Riordan *et al.* (99) suggest that arginyl residues play a unique role in anion recognition. A large diffuse cation like arginine is well suited to interact with large bio-anions like phosphates and sulfates. In modulating interactions between glycosaminoglycans (*e.g.*, heparin) and proteins, arginines are also generally preferred over lysines, with histidines being a distant third (100). This seems to be a common theme for peptides and compounds that bind to highly anionic biomolecules such as LPS and heparin.

Design of smaller nonpeptide compounds that target LPS

A few research groups have used a structure-based approach exploiting the amphipathic and positive charge character of cationic peptides and the structural limitors on interactions with groups on lipid A, *e.g.*, the phosphates of the diglucosamine moiety, to design smaller nonpeptide LPS-binding compounds that neutralize LPS. Early on, David (101) wrote a thorough review that outlines various structural/compositional characteristics necessary for the development of small-molecule antiendotoxin agents.

In one study, Guo *et al*. (102) employed *in silico* molecular modeling to analyze the interactions of biscationic ligands from a diverse library with the lipid A group of LPS, and demonstrated that the pharmacophore required for optimal interaction by small molecules contains two protonatable positive charges separated by a distance of about 14 Å. This distance is essentially that found, for example, with the two arginines separated by 13 Å in the structure of peptide LF11 complexed with LPS (89). In this regard, the electrostatic interactions with the two phosphates on lipid A were found to be the most crucial element governing efficient small molecule-mediated LPS neutralization, with hydrophobic forces being secondary. Burns *et al*. (103) exploited this observation and evaluated a designed combinatorial library to identify several relatively high-affinity amphipathic bisamide compounds. While most analogues neutralized LPS at concentrations above 10 μM, some were active at < 10 μM. The best bisamide exhibited an IC_{50} value in the single-digit micromolar range and contained a 1,3-diaminopropane, a tryptophan group and a C_{18} alkyl chain. The distance between the terminal amines was 14.7 Å.

Chen *et al*. (104) designed a series of calix[4]arene-scaffolded amphipathic compounds that had various appended chemical groups (aliphatic hydrophobic groups and hydrophilic groups, *i.e.*, amine, guanidinium and triazole groups). These compounds were designed to produce agents that mimicked the molecular dimensions and surface character and topology of segments of β-sheets or α-helices, as in βpep peptide (69, 70) or the dodecapeptide SC4 (71) LPS-neutralizing peptides. Some of these calixarene-based compounds displayed IC_{50} values in the single-digit micromolar range, some in the submicromolar range and a few in the range of 5-50 nM. A few were on a par with the LPS-binding BPI protein and polymyxin B. Three of the best compounds were also able to neutralize LPS *in vivo* in a mouse endotoxemia model.

NMR structural studies provided insight into how these calixarene-based compounds interact with lipid A (104). Figure 2 (left) illustrates chemical groups on lipid A that are most affected in terms of chemical shift changes via interaction with the calixarene compounds. These groups are proximal to the two phosphorylated glucosamine rings and the α- and β-carbons of the lipid A acyl chains. This proximity is also visualized in Figure 2 (right) in a space-filling model of the X-ray structure of lipid A (105) (pdb code 1qff), where the most chemically shifted groups are color coded and the two phosphates are positioned at the upper right and left regions of lipid A. Therefore, these calixarene compounds interact with lipid A consistent with the crucial role of LPS-neutralizing compounds and the two phosphates of lipid A, in addition to some hydrophobic-mediated interactions. Moreover, calixarenes that have guanidinium and amine groups neutralize LPS better than those with a triazole group (a histidine surrogate), which makes these compounds relatively ineffective. The distance between positively charged groups on the hydrophilic surface of calixarenes ranges from about 12 to 15 Å, which is consistent with the finding that the spatial arrangement of arginine groups of peptide LF11 is crucial for interactions with the phosphates of lipid A (89).

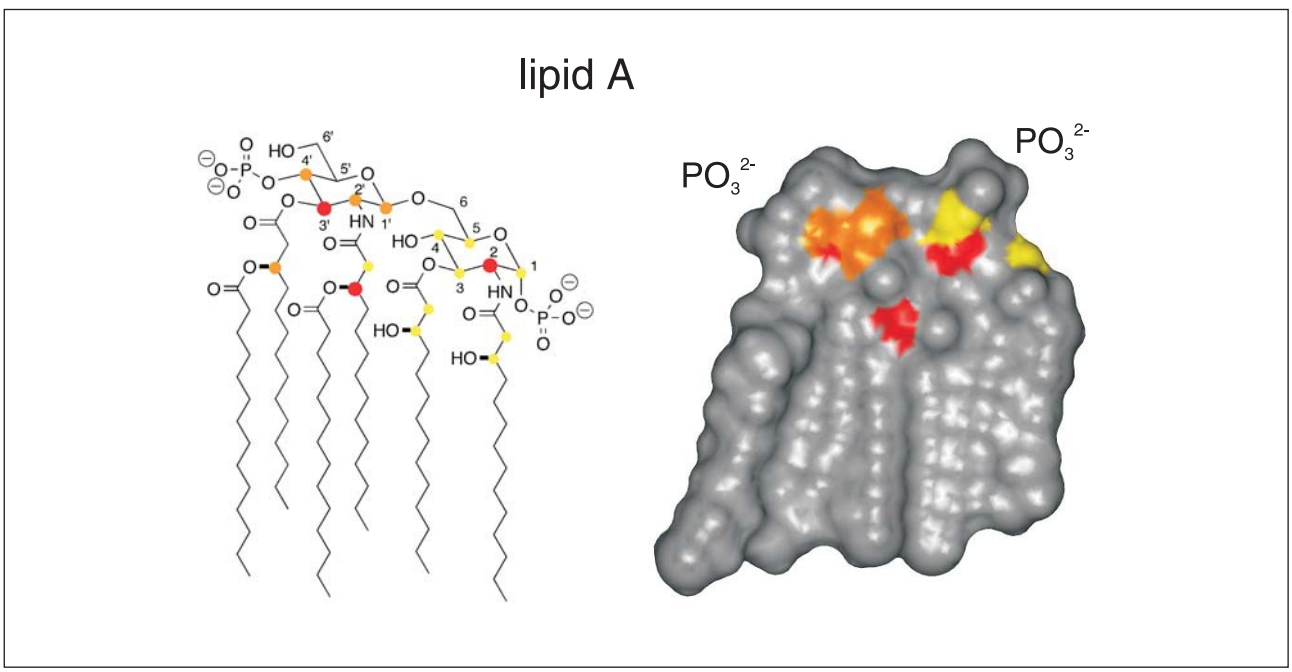

Fig. 2. The chemical structure (left) and calculated Connoly surface (right) are illustrated for hexaacyl lipid A from *Escherichia coli.* Protons most affected by the binding of designed cationic calixarene-based compounds are shown as large red circles for the most affected, and orange and yellow circles for intermediate and small chemical shift changes, respectively. The same color codes are used for the Connoly surface representation.

Towards the future

Sepsis is a very complicated pathological disorder brought on by Gram-negative bacterial infection and the release of LPS from the bacterial membrane. Because of this, there are a number of places for therapeutic intervention within the sepsis cascade, as this review has elaborated, *i.e.*, LPS neutralization, anti-cytokine immunotherapy and anticoagulation agents. Initially, antibodies against LPS and cytokines were promoted, but essentially failed in the clinic, and while anticoagulation therapy did have some clinical success, its effect was optimal only when performed in combination with other interventional therapies against sepsis. It appears likely, therefore, that any single-agent, stand-alone therapy would not likely succeed in the clinic, again due to the complex nature of the pathology of sepsis. The future lies in establishing an appropriate combination of therapeutic approaches, and it appears likely that direct neutralization of LPS using a cationic peptide will be part of this cocktail therapy approach. The use of antibiotics will kill bacteria, and yet this treatment itself leads to the release of LPS endotoxin. Using a structure-based approach to understand how agents bind to and neutralize LPS will aid in the design and optimization of LPS-neutralizing compounds. The future of antisepsis agents seems to lie both in the production of agents that can neutralize LPS at the very early preseptic stage (*i.e.*, a prophylactic agent) and in combination with other approaches to “mop up” LPS once liberated from bacterial membranes. There is hope.

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