New Insight in LPS Antagonist

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Abstract: Lipopolysaccharide (LPS) or endotoxin, the major constituent of the outer membrane of Gram negative bacteria, has been implicated as the bacterial product responsible for the clinical syndrome of sepsis. LPS binding to the host receptor Toll-like receptor 4 (TLR4) triggers an inflammatory reaction characterised by the release of large number of inflammatory mediators that allow the host to respond to the invading pathogen. When this production becomes uncontrolled and excessive, it leads to the development of septic shock. Despite decades of efforts in supporting therapies, sepsis remains the leading cause of death amongst critically ill patients. Unfortunately, the major factor contributing to the high morbidity and mortality of sepsis is the lack of the effective targeted treatment. Indeed, over 30 drugs for the treatment of sepsis have been developed: many of these target specific inflammatory mediators and have thus been, in general, unsuccessful since sepsis relies on the cross talk of several cytokines and the block of a single factor has been proven to be ineffective. More successful strategies include those modulating the early phase of LPS signalling such as the ones that prevent the binding of LPS to host cells and the subsequent cascade of detrimental events. In this light, effective LPS antagonists would represent invaluable tools to efficaciously manage sepsis. This review discusses the evolution of naturally occurring and synthetic LPS antagonists with emphasis on the development of several natural new molecules.

Key Words: Innate immunity, lipid A, lipopolysaccharide, sepsis, Toll-like receptor 4.

1. INTRODUCTION

1.1. Sepsis

Host defence against invading bacteria depends on the mobilization of cellular and extracellular antibacterial weapons to the site of invasion. This is mediated by the acute inflammatory response triggered directly and indirectly by pathogens. Lipopolysaccharide (LPS) or endotoxin, the major constituent of the outer membrane of Gram-negative bacteria, has long been known as the key bacterial product responsible for the clinical syndrome of sepsis. Sepsis and its *sequelae*, septic shock, acute respiratory distress syndrome and multiple organ dysfunction/injury, represent a continuum of a syndrome characterised by systemic inflammation and widespread tissue injury, caused by an overwhelming systemic response of the body to an infection [1]. Despite decades of efforts in supporting therapies, sepsis remains the leading cause of death among critically ill patients. There are approximately 750,000 cases of sepsis in the US annually [2, 3]. Sepsis is the cause of approximately 2% of all hospitalization and it has been estimated that there will be more than 1 million cases of sepsis per year in the US by 2020. An estimated 215,000 deaths (9.3% of all deaths) in the US occurred in patients with sepsis [4]. Unfortunately, the major factor contributing to the high morbidity and mortality of sepsis is the lack of the effective treatment. Consequently, over 30 pharmaceutical products are in development for this condition: many of these target specific inflammatory mediators and have thus been, in general, unsuccessful since the process of sepsis involves many mediators [5].

1.2. Treating Sepsis

Severe sepsis still represents a major challenge in medicine. Epidemiologic studies indicate that severe sepsis causes as many deaths annually as acute myocardial infarction. However, the understanding of the immune pathology of sepsis is still incomplete and adjuvant immune interventions (blockade of bacterial components such as LPS or host cytokines like tumor necrosis factor (TNF) and interleukin-1β have not been successful so far.

Treatment of sepsis is sub-optimal and frequently the choice of therapy is limited to broad-spectrum antibiotics. More selective treatments may be used once the pathogen has been determined; however during this delay, the clinical status of the patient frequently deteriorates to a critical condition. As an alternative to developing antimicrobial agents, researchers are focusing on therapeutics that block stimulation of host molecules, such as toll-like receptors by the microbe, thereby limiting toxicity.

A variety of approaches have been taken to alleviate the morbidity and mortality of patients due to severe sepsis and septic shock. These approaches aimed at interrupting events leading to a severe sepsis state. They include: 1) neutralizing LPS or blocking early LPS-induced signalling events; 2) blocking the intracellular signals induced by endotoxin; 3) inhibiting the release of cytokines and cellular mediators; 4) blocking the paracrine effects of inflammatory mediators produced by the infected cells. More successful strategies

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modulating entire inflammatory pathways include those that prevent the binding of LPS to host cells and the subsequent cascade of detrimental events. In this light, effective LPS antagonists would represent invaluable tools to efficaciously manage sepsis. Discussion of each of the approaches listed is beyond the scope of this review that, otherwise, will focus on the strategy of blocking LPS binding to its cell-surface receptor by the use of naturally occurring and synthetic antagonists.

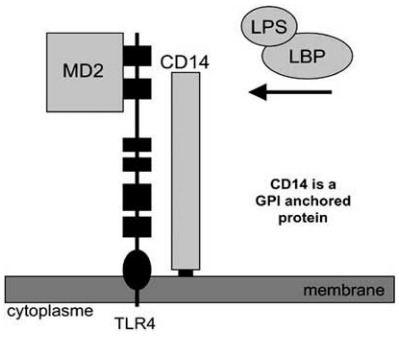

Fig. (1). TLR4 complex.

2. LPS AND LIPID A SIGNALLING

Host responses to endotoxin must be highly sensitive but self-limited and tightly controlled. Elegant studies have provided a model to the sequence of events performed by the host to detect endotoxin with extraordinary sensitivity and to "neutralize" the ability of LPS to trigger a response. In mammals, optimal sensitivity is achieved by ordered interaction of endotoxin with several different extracellular and cell-surface proteins. According to the current model, LPS initially binds to an LPS binding protein (LBP) in serum, which catalyzes the transfer of monomerized LPS from aggregate structures and in some cases from intact Gram-negative bacteria to membrane-bound CD14 (mCD14) on monocytes or monocytically-derived cells Fig. (**1**), [6-8]. mCD14 is not an integral membrane protein, but rather is bound to the membrane *via* a glycerolphosphatidylinositol (GPI) moiety, rendering CD14 unable to transduce signals across the membrane [9, 10]. Final transmembrane signalling takes place when mCD14 next transfers or presents LPS to the transmembrane toll-like receptor 4, or TLR4. This receptor works in conjunction with an obligate accessory protein MD-2, forming a TLR4/MD-2 complex that initiates intracellular signalling triggering generation and release of a wide spectrum of cytokines and cellular mediators [11-17]. LPS may also be transferred by LBP to a soluble form of CD14 (sCD14) that can transfer endotoxin to mCD14 or TLR4 directly. This complex reaction pathway also provide many ways to attenuate endotoxin-driven inflammation and can explain how differences in endotoxin structure, either intrinsic among Gram negative bacteria or induced by metabolic remodelling, can alter host responsiveness and thus the outcome of host-Gram negative bacteria interactions.

2.1. LPS and Lipid A Structures

Endotoxins are unique and very abundant surface glycolipids of Gram negative bacteria. They are amphipathic and complex molecules consisting of three distinct region: a polysaccharide (O-antigen) region, a core oligosaccharide region that includes 3-deoxy-D-manno ketodeoxyoctulosonic acid (KDO) and a conserved lipid A region that contains a β-1→6 linked disaccharide of N-acetylglucosamine linked by ester or amide bonds to 3-OH-fatty acids that may be further substituted with non-hydroxylated fatty acids in an acyloxacyl linkage, Fig. (**2**).

The lipid A region represents the main toxicophore. While endotoxin is a complex heterogeneous molecule, the toxic portion of LPS (the lipid A portion) is relatively similar across a wide variety of pathogenic strains of bacteria, making this molecule an attractive target for the development of an LPS antagonist.

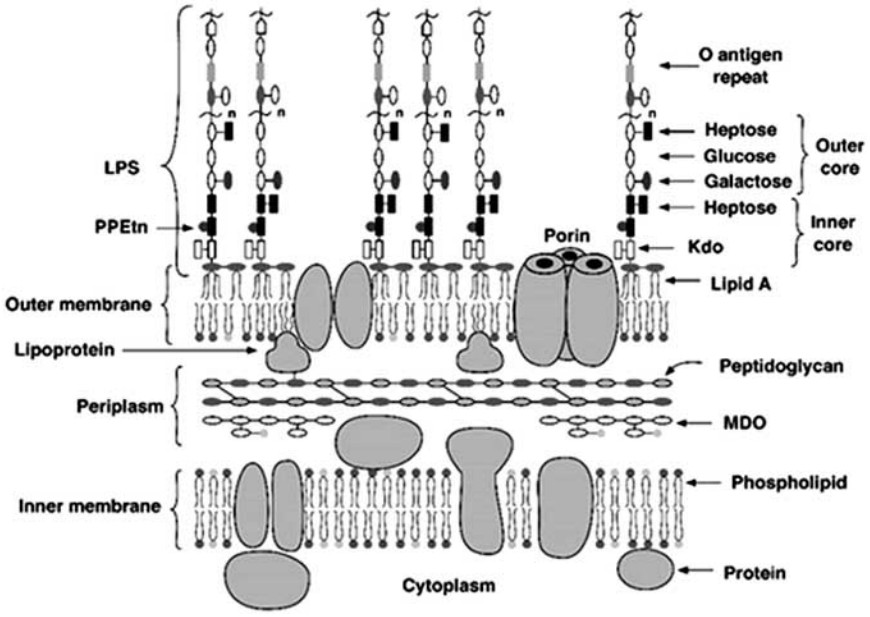

Fig. (2). Gram negative bacteria cell wall.

Lipid A from *Escherichia coli* (*E. coli),* Fig. (**3**), was first isolated by Westphal and Luderitz [18] and found to exert toxic and pyrogenic effects. Most importantly, lipid A was found to induce the release of TNF-α to an extent equal to that of the parent LPS molecule [19]. The central role that lipid A plays in activating cells prompted the hypotesis that blocking the binding to one or more of its signalling partners will block all downstream events leading to systemic inflammatory response and septic shock. Interestingly, the toxicity of lipid A was found to be directly correlated to the levels of acylation. Consistent with this, the highest level of toxicity has been attributed to *E. coli* LPS where lipid A is present in a hexa-acylated form.

Recent findings demonstrate that structurally altered lipid A are less or not agonistically active, rather acting as antagonist of *E. coli* LPS and reducing its deleterious effects. In fact, penta-acylated lipid A from two phototropic species, *Rhodobacter capsulatus* and *Rhodobacter sphaeroides* have shown antagonistic effect [20, 21]. More recently, we have also demonstrated that a structurally novel lipid A derived from *Halomonas magadiensis*, a Gram-negative extremophilic and alkaliphilic bacterium, consisting of a heterogeneous mixture of penta- and tetra-acylated lipid A, has antagonistic properties in human cells, likely due to its capability to interfere with TLR4-mediated immune activation [22]. Pharmacological receptor antagonists have been successfully shown to be derived from modification of a parent agonistic molecule [23]. With these considerations in mind, it is feasible to predict that our current knowledge together with help of mother nature will help us designing naturally occurring and chemically-modified antagonists (possibly derived from a variety of bacterial species) containing alterations of the core structure of natural lipid A.

3. LPS ANTAGONISTS

Several reports have described natural and synthetic inhibitors of LPS-induced inflammatory responses and they can be divided in two broad categories: TLR4 antagonists and LPS signalling interfering molecules [24].

3.1. TLR4 Antagonists

Among these there are LPS-like molecules isolated from bacteria like *Oscillatoria planktothrix, Rhodobacter capsulatus, Helicobacter Pylori, Porphyromonas gingivalis* and *Capnocytophaga ochracea* or synthetic lipid A analogues including CRX-526.

3.1.1. CyP

In a work by Macagno *et al.* [24], a LPS-related molecule derived from the cyanobacterium *Oscillatoria planktothrix* FP1 and termed CyP, was characterised. Such as other LPS molecules derived from cyanobacteria CyP contains glucose, xylose, mannose and rhamnose, but unlike gram-negative LPS, the presence of galactose and glucosamine is variable and both 2-keto-deoxy-octulosonic acid and heptose are either low (0.15-2% of LPS) or absent. Cyanobacterial LPS molecules also contain relatively large quantities of oleic, palmitoleic, linoleic and linolenic acids, which are typically absent in gram-negative LPS molecules. In addition, unlike lipid A from gram-negative LPS, including the meningococcal LPS, lipid A from cyanobacteria does not contain phosphorus [25].

The cyanobacterial LPS-related molecule CyP acted as a competitive inhibitor of *E. coli* LPS (Ec-LPS) binding to MD-2, the endotoxin binding unit. CyP inhibited LPS induced activation of both MyD88-dependent and -independent pathways. The MyD88-dependent pathway signals through IRAK and TRAF6, leading to activation of NF-κB and mitogen-activated protein kinases, such as p38, ERK and JNK, while MyD88-independet signalling requires activation of IRF3, necessary for the induction of IFN-β synthesis. It was assessed that CyP inhibited almost completely p38, ERK and c-Jun phosphorylation as well as nuclear transloca-

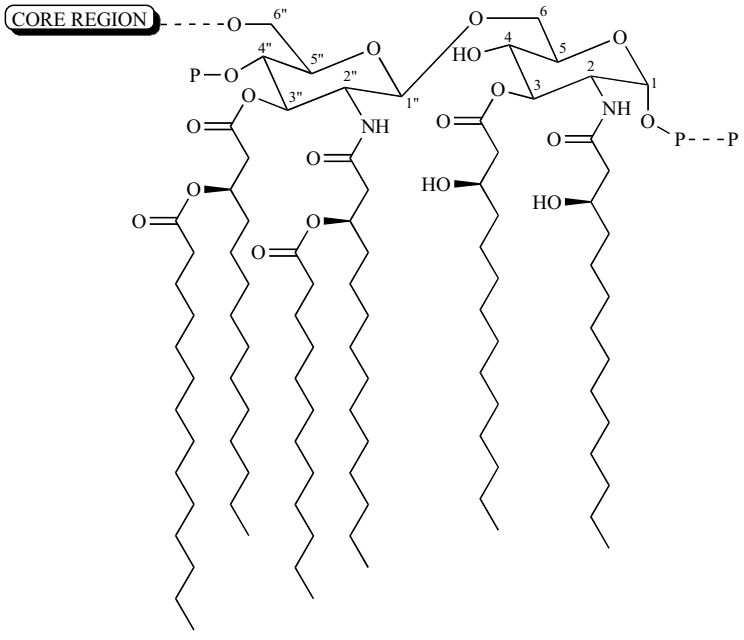

Fig. (3). Lipid A of *E. coli.*

tion of IRF 3. Moreover, CyP prevented LPS-induced IκBα degradation and subsequent induction, both indicative of NF-κB activation. A microarray analysis furtherly confirmed that CyP is a full TLR4 antagonist and inhibits the entire LPS-induced activation program impeding all intracellular responses. Significantly, CyP suppressed gene transcription and cytokine production in dendritic cells treated with Ec-LPS and also protected mice from endotoxic shock caused by *Salmonella abortus* LPS [24].

In a very recent study by Jemmett *et al.* [26] it has been shown that CyP inhibited the secretion of the pro-inflammatory cytokines IL-1β, TNF-α and IL-6 and of the chemokines IL-8 and MCP-1 induced by treatment of human blood with pure *Neisseria Meningitidis*-LPS (Nm-LPS). Cytokines inhibition was independent of meningococcal serogroup, demonstrating broad applicability and CyP itself had no effect on meningococcal viability or growth. The efficacy of CyP in the human whole blood model of septicaemia was dependent on time of administration. Furthermore, CyP was effective when added to blood within 1h of stimulation with meningococci or meningococcal components but not at later times.

Moreover, the fact that CyP similarly inhibited cytokines secretion induced by Ec-LPS, suggests that CyP acts through a common mechanism as an antagonist of the activity of gram-negative LPS. *In vitro* studies with human dendritic cells and TLR4-transfected Jurkat cells demonstrated that CyP competitively inhibited Nm-LPS interactions with TLR4 thus acting as a potent antagonist of meningococcal LPS [26].

By contrast to its effects on pro-inflammatory cytokines production, CyP alone, at the highest concentration tested (20μg/ml), induced secretion of the chemokines IL-8 and MCP-1 in whole blood. Although the CyP preparation consists mainly of a LPS-like glycolipid, which is most likely responsible for the antagonistic effects [24], it is possible that minor contaminants are responsible for stimulating chemokines expression. However, several other possibilities cannot be ruled out.

In a recent study by Thorgersen *et al.* [27], it has been demonstrated that CyP inhibited TNF-α, IL-1β and IL-8 production to basal levels in a porcine whole blood model assay. This suggests that, similarly to what has been previously observed in mice and humans, CyP interferes with LPS activity also in pigs. It is, therefore, likely that CyP inhibits LPS-induced cytokine response in whole blood through a well conserved evolutionary mechanism, thus irrespective of species.

3.1.2. LPS from Rhodobacter capsulatus

It has been shown that the nontoxic *R. capsulatus* LPS inhibited cytokine induction by three LPSs in cultured mononuclear cells (MNC) while inducing release of IL-1 and IL-6 but not of TNF only at very high concentrations (10 or ≥50 μg/ml) [20]. Further experiments analyzed whether the nontoxic *R. capsulatus* LPS could antagonize cytokine induction by LPS from *Bradyrhizobium lupini, Rhizobium meliloti* or *Pseudomonas diminuta*: coincubation with *R. capsulatus* LPS abolished the induction of IL-1 and IL-6 or of TNF by the other LPSs.

As described in Table **1**, some of these LPSs possess unusual fatty acids, such as 3-oxo-14:0, or long chain hydroxylated fatty acids, such as 27-OH-28:0, in addition to 3-OH fatty acids, as usually found in enterobacterial lipid A. Others contain lipid A with 2,3-diamino-2,3-dideoxy-D-glucose instead of glucosamine as the backbone sugar. Some lack phosphate or contain only ester-linked phosphate and lack glicosidically linked phosphate groups attached to the backbone [20].

DOC-PAGE was performed to analyze the individual migration patterns of the LPSs. The LPSs showed different patterns depending on the extent of R-core substitution by O-chains and on different O-chain composition and length. *R. capsulatus* and *R. meliloti* LPSs presented short O-specific chains. The LPS of *P. diminuta* had high rough-type content, whereas *B. lupini* contained significant amounts of smooth LPS. The minimal concentrations active for cytokine induction and the toxicity (LD_{100}) of the LPSs tested indicate that expression of biological activities is not related to the type of LPS (smooth or rough). Furthermore, the *R. capsulatus* LPS reduced or abolished cytokine induction following stimulation of MNC with the other LPSs. The overall structure of the lipid A backbone, the number and type of fatty acids,

Table 1. Chemical Characteristics of LPSs

LPS Source	Disaccharide Backbones	Phospate Linkage	Fatty Acids	
			Amide Bound	Ester Bound
Rhodobacter capsulatus 37b4	Glucosamine	Glycosidic and ester	3-Oxo-14:0, 3-OH-14:0	3-OH-10:0, partially substituted by Δ5-12:1
Pseudomonas diminuta NCTC 8545 25	2,3-diamino-2,3-dideoxy-D-glucose	Ester	3-OH-12:0 3-OH-14:0	14:0, unusual fatty acids
Bradyrhizobium lupini DSM 30140	2,3-diamino-2,3-dideoxy-D-glucose	Glycosidic	3-OH-12:0, 3-OH-14:0	16:0, 18:1, 29-OH-30:0, 30-OH-31:0
Rhizobiuim meliloti	Glucosamine	Glycosidic and ester	3-OH-16:0, 3-OH-18:0	3-OH-14:0, 27-OH-28:0

their position and complete phosphorylation all appear to be important for the expression of biological activities. The structure-function relationship of the LPSs tested supports the concept that none of the different lipid A constituents represents the toxophore group by itself. It is more likely that expression of biological activities depends on the overall structure of the lipid A and that structural similarity to enterobacterial lipid A is important. The influence of the type of acylation within the lipid A backbone is documented by the results obtained with the *R. capsulatus* and *R. meliloti* LPSs. Replacement of the amide-bound 3-OH fatty acids of *R. meliloti* by 3-oxo fatty acids, as found in the *R. capsulatus* lipid A, in concert with the presence of shorter and unsaturated fatty acids results in a 1,000-fold decrease in toxicity and 1,000- to 10,000-fold decrease in cytokine-inducing capacity. A similar observation was made earlier when the toxicity of *Rhodopseudomonas sphaeroides* LPS containing amide-bound 3-oxo fatty acids and enterobacterial lipid A containing amide-bound 3-OH fatty acids was compared [28]. Two of the LPSs tested (*B. lupini* and *R. meliloti*) contain long-chain fatty acids twice as long as those usually present in enterobacterial lipid A [29, 30]. Consequently, they contribute to a high hydrophobicity of the respective lipid A and may compensate for the low degree of acylation (4 to 5 fatty acids per molecule) of *B. lupini* and *R. meliloti* LPS. Study of synthetic lipid A partial structures showed that the fatty acid composition influences the ability of various synthetic lipid A's to induce IL-1 [31]. Induction of IL-1 by LPS or lipid A could be antagonized by non-IL-1-inducing tetraacylated precursor Ia, indicating specificity of binding, which, however, does not suffice to induce lymphokine production. Since the *R. capsulatus* LPS only killed galactosamine-treated mice and induced cytokines at high concentrations, it was analyzed whether this LPS inhibited induction of cytokine production by toxic LPSs. The *R. capsulatus* LPS did inhibit or decrease (by 45 to 99%) the lymphokine induction by all LPSs tested. These investigations agree well with findings that lipid A obtained from the LPS of *Rhodopseudomonas sphaeroides*, structurally similar to *R. capsulatus* LPS, inhibited TNF production by toxic LPS in RAW 264.7 cells [32] and that lipid IVA inhibited TNF production in whole blood *ex vivo* [33].

3.1.3. Helicobacter pylori LPS

At least ten different TLRs have been identified, which are able to recognize a diverse array of pathogen-associated molecular patterns. TLR2 has been shown to recognize peptidoglycan and bacterial lipoproteins, whereas TLR4 has been shown to recognize LPS from Gram-negative bacteria. Lepper PM and colleagues set up a study in 2005 to better clarify the role of TLR2 and TLR4 in cellular responses to *H. pylori* LPS [34]. By utilizing reporter cell lines as well as HEK293 cells transfected with specific TLRs, they demonstrated that *H. pylori* LPS-induced activation is mostly TLR2 dependent. Surprisingly the results revealed an unique ability of some *H. pylori* strains to act as antagonists for human TLR4, thus suggesting that depending on the strain, there might be differences in lipid A structure, and thus engagement of different receptors.

The addition of *H. pylori* LPS from strain HP688 attenuated the TNF-α secretion by Mono Mac 6 cells stimulated with *E. coli* LPS, thus demonstrating that the *H. pylori* LPS from this particular strain can act as an LPS antagonist. In order to further determine whether *H. pylori* LPS was a TLR4 antagonist, a CHO/CD14/TLR4 reporter cell line was used. When this cells were preincubated with *H. pylori* LPS (1 μg/ml), it resulted in an attenuated expression of CD25 on the cell surface in response to *E. coli* LPS, thus suggesting that *H. pylori* LPS specifically antagonizes TLR4. The greatest antagonistic activity was observed with clinical strain HP688.

Although TLR4 had previously been implicated in *H. pylori* LPS recognition [35, 36], Lepper suggested that responses against *H. pylori* LPS are mediated *via* TLR2, confirming a previous study from Smith *et al.* [37] who demonstrated that TLR2 and not TLR4 is required for *H. pylori*-induced NF-κB activation.

The differences in TLR4 antagonistic activity observed among the ten clinical strains analyzed must be attributed to differences in lipid A structure. It has been previously demonstrated that fresh clinical isolates of *H. pylori* produce high molecular-weight smooth types of LPS with long O side-chains [38, 39]. However, *H. pylori* strains that have been subcultured may produce low-molecular-weight rough-form LPSs which lack the O side-chain [40]. A few years ago, Moran *et al.* [41] shed more light into the actual structure of *H. pylori* LPS. They have demonstrated that the lipid A component of *H. pylori* LPS is a mixture of predominantly tetraacyl variety and a minor hexaacyl form. Different *H. pylori* strains might have LPSs containing varying amounts of either tetraacyl or hexaacyl lipid A, resulting in a different LPS 'shape' and thus a different response. In particular, they postulated that clinical strain HP688 might have more tetraacyl lipid A, and thus is the most potent TLR4 antagonist from all the strains that they tested. LPSs that have a tetraacyl lipid A, such as LPS from *R. capsulatus*, *R. sphaeroides* or synthetic compound 406, have been shown to assume a more 'cylindrical shape' which results in higher antagonistic activity [42]. HP688 must be more cylindrical than the other *H. pylori* clinical strains and thus it has the highest antagonistic activity. Activation *via* TLR2 results in a subdued inflammatory response [43] allowing the organism to establish a chronic foothold in the gastric epithelium. Finally, the TLR4 antagonistic activity that some strains seem to have might provide the particular strain with a great advantage over the host in order to escape from the host innate immune system. The variation in antagonistic activity could possibly be a virulence factor for certain strains, and might be associated with the progression of *H. pylori*-associated diseases.

3.1.4. LPS from Porphyromonas gingivalis and Capnocytophaga ochracea

Other interesting natural LPSs that have shown antagonistic effect on human TLR4 are the LPSs from *Porphyromonas gingivalis* and *Capnocytophaga ochracea*.

In fact, in an elegant study by Yoshimura *et al.* [44], it has been demonstrated that the expression of CD25 induced by stimulation of 7.7/huTLR4 cells (lacking TLR2) with LPS either from *E. coli* or *Actinobacillus actinomycetemcomitans* or *Fusobacterium nucleatum*, was attenuated by the

addition of LPS from *P. gingivalis* or *C. ochracea.* On the other hand, these LPSs were not able to inhibit *Sthaphylococcus aureus*-induced CD25 expression in 7.7/huTLR2 cells (not expressing TLR4). Furthermore, *E. coli*, *A. actinomycetemcomitans* or *F. nucleatum* LPS-induced IL-6 secretion by U373 cells (which have been reported to expresses TLR4 but not TLR2 [45]) was attenuated by the addition of LPS from *P. gingivalis* or *C. ochracea*, indicating that both LPS worked as antagonists in this cell line. Their data clearly indicate that LPS from *P. gingivalis* and *C. ochracea* inhibit TLR4-mediated signalling but not TLR2-mediated signalling.

The analysis of the chemical structure of *P. gingivalis* lipid A revealed the absence of ester-linked phosphate at the 4' position of glucosamine disaccharide and the presence of branched and relatively longer fatty acids, features probably responsible of the antagonistic activity [46, 47].

In contrast to 7.7/huTLR4 and U373 cells, human PBMC and gingival fibroblasts were weakly activated by LPS from *P. gingivalis* and *C. ochracea*, consistent with previous reports by other investigators [48-50]. Since those cells express both TLR2 and TLR4, the TLR2-signalling complex might be activated in these cells. This could be accounted to small amounts of contaminants that may have remained in *P. gingivalis* and *C. ochracea* LPS preparations which might activate human PBMC and gingival fibroblasts through TLR2.

Yoshimura and colleagues also demonstrated that the overexpression of human TLR4 in CHO/CD14 cells, which express a functional hamster TLR4, abrogated the sensitivity to LPS from *P. gingivalis* and *C. ochracea*, indicating that these LPSs are antagonists for human TLR4 but not for hamster TLR4. It has been suggested that the species-dependent discrimination of lipid A substructures is fully attributable to the species origin of TLR4. The antagonistic activity would be a great advantage for the microorganisms to escape from the innate immune system.

3.1.5. CRX-526

Fort *et al.* developed a lipid A mimetic peptide called CRX-526, also known as an aminoalkyl-glucosamine-phosphate [51] Fig. (**4**).

CRX-526 blocked LPS ability to trigger proinflammatory responses from human monocytes both *in vitro* and *in vivo* in two mouse models of colonic inflammation.

Microarray studies confirmed that this peptide acted as a pure antagonist and did not induce any gene transcription by signalling trough the TLR4 receptor complex: indeed, pretreatment of human monocytes with increasing amounts of CRX-526 before exposure to LPS completely suppressed all LPS-induced gene transcription.

Although the precise mechanism of action of CRX-526 is still under investigation, Fort and colleagues hypothesized that this peptide binds directly to the TLR4 receptor complex and sterically inhibits the ability of LPS to bind. It is also possible that CRX-526 directly binds to a TLR4-MD-2 complex.

This antagonistic effect is strictly dependent on the molecule structure: longer hexanoic fatty acyl chains (SAC) led to an impairment of its antagonistic activity and an increase in agonist activity for the TLR4 receptor complex. Moreover, its antagonistic activity directly depends on the presence of SAC in the left and middle positions of the molecule.

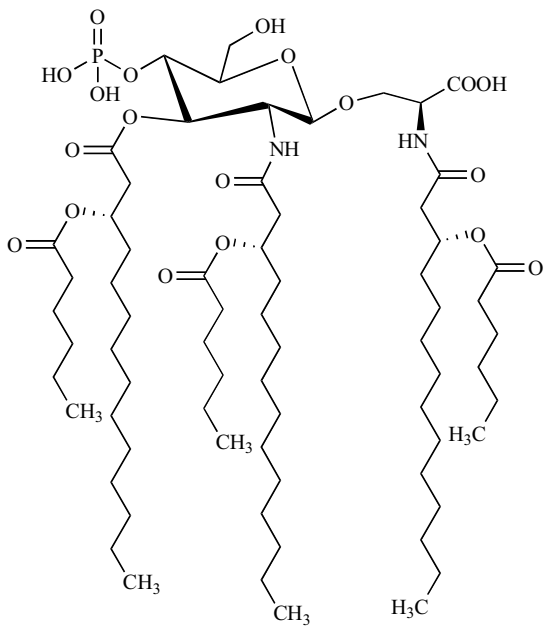

Fig. (4). Chemical structure of CRX-526.

3.2. LPS Signalling Interfering Molecules

These agents cannot be defined as LPS receptor antagonists but they may rather act intracellularly either upstream or downstream in TLR4 signalling thus behaving as functional antagonist of LPS-induced biological effects. LPS signalling interfering molecules include synthetic peptides and organic molecules.

3.2.1. Peptides of CD200

Cells of the myeloid lineage display a family of receptors, the triggering receptors (TREMs), also expressed on myeloid cells, which play an important role in regulation of acquired and innate immunity, and inflammation. Gorczynski *et al.* characterised a member of the TREM family, CD200R [52]. CD200 regulates inflammation in a number of tissues and model systems [53-59]. A viral homologue of CD200 has also been shown to modulate both macrophage and mast cell activation following interaction with CD200R1, as well as viral immunity to human herpesvirus 8 (Kaposi's sarcoma-associated herpesvirus) [60, 61]. The onset of a generalized, often lethal, inflammatory state associated with surgical sepsis is now believed to follow in part from activation of pattern recognition receptors of the innate immune system, thus focusing attention on the mechanisms involved in the suppression of non-specific inflammation *per sé* [62].

Peptides defining the CDR1 (#4004 and 6061) or CDR3 (#4013 and 4006) regions, or mapping to the C" (#6062) and F (#6059) "faces" of the 3-D CD200 structure [63] that interact with CD200R1 have no direct agonist activity. However, these latter peptides did exhibit antagonist activity.

Since CD200-derived peptides have profound anti-inflammatory effects, it was investigated whether they might function *in vivo* in assays exploring both mortality and TNF-α secretion into serum following LPS injection. In wild-type mice, both CD200Fc and peptide #4005 protected animals from death following LPS administration and peptide #4005 was effective in suppressing LPS-induced TNF-α production *in vivo* while #4004 and #4013 were found to block CD200Fc-mediated suppression of LPS induced TNF-α production.

3.2.2. TREM Peptide

TREM-1 is a recently identified molecule involved in monocytic activation and inflammatory response [64, 65]. It belongs to a family related to NK cell receptors that activate downstream signalling events. The expression of TREM-1 on PMNs and monocytes/macrophages has been shown to be inducible by LPS [66, 67]. Based on the TREM-1 sequence, a peptide called LP17 was synthesized by Gibot and colleagues and its activity on sepsis was analyzed [68].

The inducible release of proinflammatory cytokines was significantly lower after LPS stimulation when the medium was supplemented with LP17 or IL-10. LP17 reduced, in a concentration-dependent manner, the TNF-α and IL-1β production from cells cultured with LPS or with LPS and mAb and simultaneously increased the release of soluble TREM-1 (sTREM-1) from cells cultured with LPS. LP17 inhibited the NF-κB activation induced by the engagement of TREM-1 but did not alter the effect of LPS.

Furthermore, mice treated by a single dose of LP17 1 hour before a lethal dose of LPS were prevented from death in a dose dependent manner and a delayed treatment up to 4 hours conferred significant protection against an LD_{100} dose of LPS. No late death occurred over 1 week, indicating that LP17 did not merely delay the onset of LPS lethality but provided lasting protection.

LP17 comprises the complementary determining region-3 and the "F" β strand of the extracellular domain of TREM-1. The latter contains a tyrosine residue mediating dimerization. Radaev *et al.* postulated that TREM-1 captures its ligand with its complementary determining region-equivalent loop regions [69]. Thus, LP17 could impair the TREM-1 dimerization and/or compete with the natural ligand of TREM-1. Moreover, the increase of sTREM-1 release from monocytes mediated by LP17 could prevent the engagement of membrane TREM-1, sTREM-1 acting as a decoy receptor, as in the TNF-α system [70, 71]. Activation of the transcription factor NF-κB is a critical step in monocyte inflammatory cytokine production after exposure to bacterial *stimuli* such as LPS [72]. Among the various NF-κB/Rel dimers, the p65/p50 heterodimer is the prototypical form of LPS-inducible NF-κB in monocytes [73]. LP17 abolishes the p65/p50 NF-κB overactivation induced by the engagement of TREM-1. This might at least partially explain the effects of LP17 on cytokine production and the protection from lethality shown here to occur when the peptide was injected 1 hour before LPS-induced septic shock, or even up to 4 hours after. The modulation of TREM-1 signalling reduces, although without complete inhibition, cytokine production and protects septic animals from hyper-responsiveness and death. Modulation of TREM-1 engagement with such a peptide as LP17 might be a suitable therapeutic tool for the treatment of sepsis, particularly because it seems to be active even after the onset of sepsis after infectious aggression.

Considering that TREM-1 expression is increased during sepsis at both mRNA and protein levels [64, 74, 75] and that TREM-1 binding to its unknown ligand synergizes the effects of endotoxins, amplifying the synthesis of proinflammatory cytokines such as TNF-α and IL-1β and inhibiting IL-10, the blunting of TREM-1 expression appears an interesting approach [65, 76]. Gibot and colleagues have recently published another paper demonstrating that partial silencing of TREM-1 in mice subjected to a bacterial peritonitis model produced a significant survival benefit [77].

Only partial silencing of the TREM-1 mRNA conferred benefit in a polymicrobial mouse model of peritonitis, probably through blunting of the systemic inflammatory response, whereas complete silencing of TREM-1 resulted in 100% lethality, related to an impaired capacity of neutrophils to generate an adequate respiratory burst to remove bacteria. By contrast, TREM-1 silenced mice were highly resistant to a lethal endotoxin challenge where no bacteria were present, indicating a beneficial role of TREM-1 during a non-bacterial form of shock. There was a 30-40% decrease in cytokine response in less potently silenced animals during peritonitis, indeed modulation rather than abrogation of the TREM-1 pathway proved highly beneficial and resulted in a survival advantage.

3.2.3. TAK-242

To discover new small molecule inhibitors of multiple inflammatory mediator production from mouse macrophages stimulated with LPS, a screening of the Takeda chemical library was carried out and a novel cyclohexene derivative bearing a phenylsulfamoyl and ethyl ester group was identified Fig. (**5**) [78].

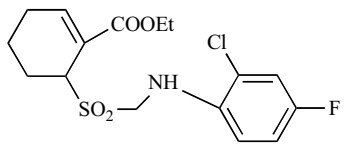

Fig. (5). TAK-242.

The R enantiomer called TAK-242 has been recently evaluated in a paper by Sha T *et al.* [79]. The authors demonstrated that TAK-242 suppressed LPS-induced production of multiple inflammatory mediators including cytokines and NO both *in vitro* and *in vivo*. In addition, TAK-242 markedly inhibited LPS induced increases in serum levels of alanine aminotransferase, total bilirubin and blood urea nitrogen, inhibited LPS-induced hypothermia and protected mice from death in a mouse endotoxin shock model. These results, therefore, suggest that the protective effect of TAK-242 is due to suppression of organ dysfunctions attributable to the inhibition of multiple mediator production.

TAK-242 inhibited the TNF-α production stimulated with the TLR4 ligands, LPS and paclitaxel, in a concentration-dependent manner. In contrast, TAK-242 did not inhibit

the TNF-α production induced by ligands for other TLRs such as Pam3CSK4, peptidoglycan, poly (I:C), loxoribine or CpG DNA.

Importantly, TAK-242 suppressed cytokine production, increase in markers of hepatic and renal dysfunction, and hypothermia, and consequently improved survival, even when it was administered 2 hours after LPS challenge, suggesting that TAK-242 may improve hepatic and renal dysfunction and prevent LPS-induced lethality even when treatment begins after the onset of organ dysfunction. The mechanisms by which TAK-242 showed significant beneficial effects even by delayed treatment are not clear. It is possible that, being a small molecule with molecular weight of approximately 360 [80], it quickly distributes tissues and mitigates inflammation at the sites as well as in blood.

Human TLR4 is homologous to mouse TLR4 (67% identical, 79% similar at the amino acid level) [81], and TAK-242 had similar inhibitory effects on cytokine production in mouse and human primary monocytes/macrophages. It is not clear how TAK-242 inhibits TLR4 signal transduction. Because TAK-242 did not inhibit LPS binding to cells and its effects with various TLR4 ligands were similar [80], it is likely that this agent does not act in a process of ligand binding on the cell surface; rather, it may act intracellularly. In addition, LPS-induced phosphorylation of mitogen-activated protein kinases, induction of cytokine mRNA, and various kind of cytokines are inhibited by similar concentrations of TAK-242 [80]. Thus, the authors speculate that TAK-242 may work upstream in TLR4 signalling. Although the molecular target of TAK-242 also remains unidentified, it is possible that TAK-242 suppresses TLR4 itself or a molecule which is specifically involved in TLR4 signalling such as MD-2 and Trif-related adaptor molecule [82, 83], because its action is TLR4 selective. Oligomerization of TLR4, and association of adaptor molecules and MD-2 to TLR4 are essential for activation of TLR4 signals [82-84]. TAK-242, however, may not directly inhibit interaction of these molecules. Because TAK-242 is a small molecule, it is unlikely that it directly inhibits interaction of large proteins. TAK-242 may affect conformational change of TLR4 that occurs after LPS binding [85]. Another possible mechanism is inhibition of phosphorylation of TLR4 and/or Trif-related adaptor molecule, which are also a key step for signal transduction upstream in TLR4 signalling [86, 87]. The precise mechanism of its action is currently under investigation.

3.2.4. Besifloxacin

Besifloxacin - Fig. (**6**), {7-[(3R)-3-aminohexahydro-1H-azepin-1-yl]-8-chloro-1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-3-quinolinecarboxylic acid}, is a novel fluoroquinolone being developed by Bausch & Lomb for the topical treatment of ophthalmic infections.

In a recent study, Zhang and Ward demonstrated that besifloxacin significantly inhibited LPS-induced cytokine production in THP-1 monocytes and that these cytokines could be divided into two different response groups: the first group of cytokines includes GM-CSF, IL-1β, IL-8, IP-10, MCP-1 and MIP-1α. For these cytokines, both moxifloxacin and besifloxacin had comparable effects after LPS stimulation. The second group of cytokines includes those for which besifloxacin demonstrated either better (G-CSF, IL-1α, IL-1ra, IL-6 and VEGF) or less (IL-12p40) potency than moxifloxacin [88]. The molecular mechanisms underlying these effects are not clear, even if inhibition of MAP kinase and NF-κB activation has been reported for moxifloxacin in monocytes and cystic fibrosis epithelial cells [89].

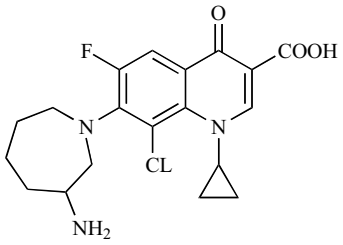

Fig. (6). Besifloxacin.

3.2.5. Compound K

In a recent work by Yang, *et al.*, it has been demonstrated that Compound K (C-K) Fig. (**2**), a metabolite produced by intestinal bacteria in humans and rats from the protopanaxadiol ginsenosides Rb1, Rb2 and Rc, regulates distinct TLR4-mediated inflammatory responses [90].

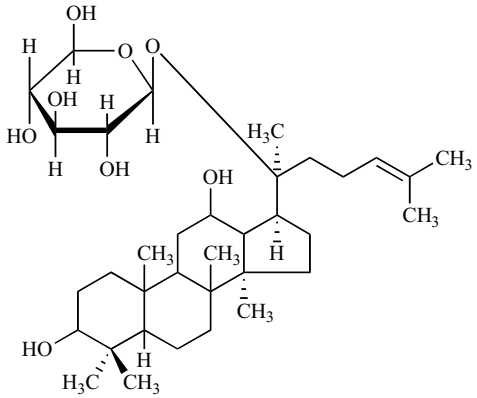

Fig. (7). Compound K.

Both TLR2- and TLR4-mediated inflammatory responses through MyD88-dependent pathways are attenuated by C-K, which functions as an agonist ligand for the glucocorticoid receptor (GR). C-K failed to inhibit TLR3-dependent signalling, further supporting the TLR/GR cross-talk model in which GR ligands modulate the TLR signal through MyD88-dependent, but not Trif-dependent, pathways [91]. Interestingly, C-K attenuated the production of proinflammatory cytokines induced by HMGB1, a therapeutic target for the treatment of lethal systemic inflammation. Recent studies reported that HMGB1 induced the inflammatory signals through TLR4 and TLR2 [91]. This indicates that the ginsenoside metabolite C-K regulates MyD88-dependent signalling *via* GR engagement. Negative regulation of inflammatory responses is thought to result, at least in part, from the ability of GR to interfere with the activities of other signal-dependent transcription factors, including NF-κB and AP-1 family members, *via* transrepression [92].

C-K attenuates proinflammatory cytokine production by macrophages and protects septic mice against hyperresponsiveness and death. Of note, postinjection of C-K significantly increased the survival rate of septic mice. Moreover, C-K was effective in a sepsis model that included cecal ligation and puncture, which closely mimics human acute peritonitis and is regarded as the most clinically relevant animal model of sepsis. Taken together, these data suggest that C-K may be useful as a therapeutic tool for the treatment of sepsis by altering the inflammation induced by TLR4.

CONCLUSION

Research over the past fifteen years focused on the design of various lipid A analogs including monosaccharide, acyclic and disaccharide compounds has lead to the development of several highly potent LPS antagonist. Two generations of synthetic analogs of antagonistic lipid A have been designed to antagonize the effect of endotoxin. The first generation exponent E5531, an analog of the lipid A from *Rhodobacter capsulatus*, demonstrated potent inhibition of LPS *in vitro* and *in vivo* but its activity decreased as a function of time for the interaction with plasma lipoproteins [93, 94]. Superior activity and pharmacological characteristics have been shown by E5564 [95], a second-generation LPS antagonist derived from the structure of *Rhodobacter sphaeroides*.

The specific TLR4 antagonist, E5564, commercially known as Eritoran, is now in clinical trials to determine the potential efficacy of TLR4 inhibitors in septic shock. Eritoran has recently been tested in a double-blind, placebo-controlled human study, and did indeed block the toxic effects of an endotoxin infusion in healthy volunteers [96]. Fever, tachycardia, leukocytosis, rise in C-reactive protein and measurements of TNF-α and IL-6 were significantly different in individuals receiving Eritoran than in those receiving placebo. Subjective symptoms such as nausea, headache and myalgia were also minimised. No agonist effect has been observed with this compound. This TLR4 inhibitor has successfully completed phase II clinical trials in septic patients and has now entered a phase III clinical program, results are expected for the end of 2009 [97]. Another potential therapeutic agent that affects TLR4 signalling is TAK-242. This molecule has completed phase III clinical program for severe sepsis on February 2007 [97].

Despite advances in diagnostic techniques, surgical therapy, intensive care treatment and use of potent antibiotics, serious gram-negative bacterial infection in conjunction with the sepsis syndrome is associated with significant risk of mortality (about 30-50%) [98]. LPS is a major toxin in patients undergoing cytoreductive chemotherapy for cancer, as is invasion by Gram-negative organisms or their cell wall components across a damaged gut mucosa. LPS is also a major stimulus for reactive airways diseases, including asthma and chronic obstructive pulmonary disease (COPD) [99] and obstetric dysfunction in "ripening" of the cervix and preterm labour [100]. The ability to block LPS signalling in these situations may prove helpful. The development of new therapies for the treatment of Gram-negative bacterial sepsis has been the focus of extensive investigation. Molecular and cellular biologic techniques have led to important advances including identification of naturally occurring LPS-binding protein, generation of novel LPS-binding antibodies, proteins and peptides and characterization of the molecular determinants of LPS binding. Taken together these advances can be expected to further the development of the next generation of novel, adjuvant therapies for the treatment of sepsis syndrome caused by gram-negative bacterial infection and endotoxemia.

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