IN VITRO ASSESSMENT ON THE ABILITY OF A NOVEL LIPOPOLYSACCHARIDE BINDING COMPOUND (EVK063) TO INHIBIT CYTOKINE PRODUCTION IN LPS-STIMULATED EQUINE PERIPHERAL BLOOD MONONUCLEAR CELLS

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Abstract

Objective: To assess the *in vitro* ability of a novel lipopolysaccharide binding compound (EVK063) to inhibit cytokine production in lipopolysaccharide-stimulated equine peripheral blood mononuclear cells

Animals: Eight healthy horses were sources for mononuclear cells.

Procedures: Replicate aliquots (concentrated at 4-5 million cells/mL) were stimulated with *S. typhimurium* lipopolysaccharide (LPS) (100ng/mL), treated with graded concentrations of EVK063, (0.01μM, 0.1μM, 1μM, 10μM), Polymyxin B (PMB) (10μM) and incubated at 37°C for 6 hours. Media and cell samples were collected and stored at -80°C for evaluation of Tumor necrosis factor (TNF) using an equine specific ELISA and Interleukin-6 (IL6) via qRT-PCR. NanoDrop confirmed RNA quantity and primer sets designed for equine IL6 and the housekeeping gene 18s were used. EVK063 toxicity was evaluated with propidium iodide staining as determined by flow cytometry. Data was normalized, expressed as percent inhibition of cytokine up-regulation by LPS, and statistically evaluated by analysis of variance. Statistical significance was set at $P \leq 0.05$.

Results: Samples incubated in media with 0% serum demonstrated the following results: 0.01μM and 0.1μM EVK063 maintained >90% cellular viability yet failed to significantly inhibit TNF production or IL6 expression. The 1μM and 10μM EVK063 concentrations exhibited 25% and 70% cell death respectfully and therefore an interpretation as to their efficacy to inhibit TNF production or IL6 expression could not be made. Samples incubated in media with 10% serum demonstrated the following

results: 0.01μM, 0.1μM and 1μM concentrations of EVK063 maintained >90% cellular viability yet failed to inhibit TNF production or IL6 expression. The 10μM EVK063 concentration exhibited 35% cell death and therefore an interpretation as to the efficacy to inhibit TNF production or IL6 expression could not be made. In a whole blood preparation, all samples evaluated maintained >90% cellular viability. The 10μM EVK063 significantly reduced TNF production and IL6 expression.

Conclusion: This *in vitro* study confirms the ability of EVK063 to inhibit TNF production and IL6 expression in LPS stimulated equine mononuclear cells with comparable results to PMB.

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Chapter 1

Background literature

History

Endotoxemia is a devastating clinical condition in human and veterinary medicine. Despite constant medical advancements, endotoxemia remains a leading cause of death in the horse, and the number one non-coronary cause of death in the human intensive care unit accounting for greater than 215,000 deaths annually.[1] The disease process overwhelms the hosts defense mechanisms and promotes a viscous inflammatory response responsible for the associated clinical signs. One reason for the high mortality rate is the inability to reduce the inflammatory response due to the lack of appropriate non-toxic antiendotoxic therapy. Consequently, research has focused on the cause of these clinical signs, their pathogenesis and potential attenuation of their effect. As a result, valuable data has been generated that began over 100 years ago in Germany and continues even now, with investigations going on worldwide.

Richard Pfeiffer, a German physician alongside Robert Koch, was working with the organism vibrio cholera when he made two important observations.[2,3] Pfeiffer noticed the organism released two toxins, an exotoxin when alive and a heat stable toxin after death.[2,3] He believed the heat stable toxin was sequestered by the organism while alive and therefore labeled it endotoxin. Interestingly, Eugenio Centanni was making a similar discovery in Europe. Centanni extracted a heat stable toxin from *Salmonella typhi* and named it pyrotoxina for its ability to induce fever.[2,3] A great deal of work was done through the next decade analyzing these bacterial extracts. However, it was not until the 1920's and 30's that chemists determined the bacterial extracts were complex structures

composed of polysaccharide, phospholipid and protein.[2,3] This discovery drove investigations into the relationship between the endotoxin structure and its associated clinical signs; such as hypothermic extremities, profuse sweating, weak peripheral pulses, and purple and congested mucous membranes.[2] Further interest in septic shock was stimulated by the increased incidence of sepsis and death from traumatic wounds in human medicine associated with World War II.

Investigations focusing on the mechanisms responsible for trauma induced shock and effects of bacterial infections continued in the 1950's and 60's.[4] Animal models were utilized to reproduce clinical effects previously observed in human patients suffering from sepsis and shock. Studies were conducted at Harvard University and the University of Minnesota where cardiac parameters were evaluated when anesthetized canines were intravenously administered endotoxin.[4-6] A decrease in cardiac output, systemic blood pressure and venous return leading to poor tissue perfusion and death were demonstrated.[4-6] These findings supported the association between hypotension and poor clinical outcome in human patients with circulatory shock.

With research demonstrating similar clinical signs in multiple species, endotoxins became implicated in conditions other than traumatic wounds and septic shock. Rooney et al described 'colitis X' in horses in 1963, where endotoxins were hypothesized to cause the clinical findings of fever, dehydration, diarrhea and colic.[7] Carroll et al corroborated this hypothesis in 1965 after intraperitoneal injection of endotoxin at 0.025mg/kg created similar clinical signs including diarrhea, hemoconcentration, severe leukopenia and death.[8] They also demonstrated a species difference in the susceptibility to endotoxin. The 0.025mg/kg used in horses was much lower than the 20mg/kg used in

mice, 0.48mg/kg in cats and 0.12mg/kg in cattle.[8] The recognition of specie specific sensitivity led investigations to explore this phenomenon.

Subsequent studies contributed knowledge regarding the body's response to various amounts of endotoxin, including the synthesis and release of histamine, catecholamines, coagulation factors and arachidonic acid metabolites.[9-11] Studies using anesthetized ponies where *E. coli* endotoxin was administered at different dosages (typically 150-200 ug/kg), routes (intravenous and intraperitoneal) and concentration profiles (bolus or slow infusion) showed arterial hypoxemia, pulmonary hypertension, lactic acidosis, hypotension, leucopenia, hemoconcentration and decreased central venous pressure.[12-14] Additional *in vitro* and *in vivo* studies over the last two decades have evaluated host responses to systemic effects of low dose of endotoxin administration (20-30 ng/kg). In addition, investigations have characterized the structure of endotoxin, cellular activation pathways, specific inflammatory mediators and clinical changes associated with host exposure to endotoxin.[15-32] The information gained from these studies has proven vital towards the development of therapeutics, which are directly impacted by determination of the LPS molecule.

Structure of Lipopolysaccharide

Our understanding of the structure of the lipopolysaccharide molecule (LPS) and basic mechanisms underlying the cellular response to LPS has increased vastly in recent years (**Fig. 1.1**). Lipopolysaccharide consists of a polysaccharide portion and a lipid called lipid A (**Fig. 1.2**). The polysaccharide portion consists of a polymer of repeating oligosaccharide units, the composition of which is highly varied among Gram-negative bacteria.[33-36] A relatively well-conserved core hetero-oligosaccharide covalently bridges

the polysaccharide with lipid A.[33-36] The structurally highly conserved lipid A is the active moiety of LPS.[33-36] In order for LPS to exert its toxic effect, it must be transported to its effector cell. Lipopolysaccharide is hydrophobic and tends to form aggregates in plasma that either interact with lipopolysaccharide binding protein (LBP) or high density lipoproteins forming an endotoxin monomer.[37-41]

Lipopolysaccharide Binding Protein

Lipopolysaccharide binding protein (LBP) is synthesized by the liver and normally exists in small quantities in plasma. As part of the acute phase response, the production of LBP is increased roughly 100 fold.[41-43] Lipopolysaccharide binding protein binds to lipid A facilitating endotoxin monomer interaction with peripheral blood monocytes and macrophages.[2,39,41,44-47] As part of its function in shuttling endotoxin to the inflammatory cells, LBP increases the sensitivity of the animal to endotoxin. The LPS-LBP complex is transported to mononuclear phagocytes with a CD14 receptor on their surface.[39,42,45,48-52]

CD14

CD14 is a glycoprotein cell surface receptor that is attached to the cell via a carbohydrate linkage and whose expression is increased after LPS stimulation.[39,42,45,48-52] There are two forms of CD14; membrane bound and soluble.[53] Soluble CD14 is able to extract LPS from the blood and stimulate cells that do not contain the membrane form of CD14.[53] However, CD14 does not contain a transmembrane domain and therefore, the LPS-LBP-CD14 complex must interact with another receptor, Toll-like receptor 4 to exert its effect.[54]

Toll-like Receptors

The innate immune system recognizes bacterial products and complexes through pattern recognition receptors known as Toll-like receptors that bind and are activated by various ligands.[50,55-57] Toll-like receptors are named due to their similarities with Toll, a plasma membrane receptor discovered in the fruit fly *Drosophila melanogaster*.[58-60] Toll was determined to play an integral role in the immune function of *Drosophila* and later shown to contain a cytoplasmic domain homologous to the mammalian interleukin-1 receptor protein.[58-60] There are currently thirteen TLR's mapped between humans and mice, with most mammals exhibiting between 10-15 TLR's. Toll-like receptor 4, a type 1 transmembrane protein characterized by 22 leucine-rich repeats on its extracellular domain, was first characterized in man.[61] Subsequent studies demonstrated TLR4's ability to initiate an immune response.[62-65] While some debate existed whether TLR2 or TLR4 elicited the necessary response in the LPS pathway, studies have shown that TLR4 is required during LPS challenge.[65-69] This work confirmed that TLR4 is a principle LPS-signal transduction molecule. Research has also demonstrated the need for a co-receptor to completely activate TLR4.[61,70,71] This receptor is Myeloid Differentiation Factor 2.[61,70,71]

Myeloid Differentiation Factor 2

Myeloid Differentiation Factor 2 (MD2), a 160 amino acid secreted glycoprotein located on the cell surface, is required for recognition and signaling of LPS.[61,70,71] The LPS complex is presented to MD2 where binding to the lipid A portion occurs.[71,72] The LPS bound MD2 behaves as an activating ligand for TLR4 to form a TLR4-MD2 heterodimer.[71,72] Through these interactions, LPS is able to translocate from the cell

surface to the cytoplasm. After translocation, LPS initiates a cascade of events eventually leading to the production of multiple cytokines.

Nuclear Factor kappa B

The LPS complex interacts with a universal adaptor gene (Myeloid Differentiation Primary Response Gene 88) that links the complex to signaling molecules with the purpose of activating the Nuclear Factor kappa B (NFkB) pathway.[53]

Nuclear Factor kappa B is a transcription factor protein complex that is involved in cellular responses to stimuli, including viral and bacterial antigens such as LPS.[53,62,73] Nuclear Factor kappa B naturally exists in an inactive state as a dimer bound by Inhibitor kappa B (IkB) within the cytoplasm.[53,62,73,74] The IkB family of proteins contain multiple copies of a sequence called ankyrin repeats which allow them to mask the nuclear localization signals (NLS) of NF-κB proteins and keep them sequestered in an inactive state in the cytoplasm. Within this family, IkB-β is the most prominent isoform in the horse.[53] In order to activate NFkB, the IkB must be phosphorylated by IkB kinase (IKK).[53,62,73,74] Following activation, IkB kinase phosphorylates IkB leading to ubiquitization and eventual degradation by proteasome.[53,74] After degradation of IkB, NFkB is released from its inactive state and translocates to the nucleus where it activates expression of several kB genes.[53,74] These genes code for IkB, creating a positive feedback loop, and a variety of cytokines.[53,74] These cytokines are responsible for the clinical signs of endotoxemia.[53,74]

Cytokines

Cytokines are small protein and glycoprotein molecules that function in both the innate and adaptive immune responses. While typically host protective, during some disease

processes production is amplified and clinical effects can be deleterious. Cytokines such as Tumor Necrosis Factor-α (TNF) and IL6 are confirmed in the pathogenesis of endotoxemia and the focus of many research endeavors. Tumor Necrosis Factor and IL6 are synthesized by mononuclear phagocytes and are responsible for many of the clinical signs associated with endotoxemia. The upregulation of TNF has shown significant association with increased temperature, heart rate, lethargy and decreased white blood cell count, hypotension, hemoconcentration and endothelial dysfunction, while IL-6 correlates with enhanced synthesis of acute phase proteins, cellular differentiation and increased temperature.[16,17,24,26,31,32,46,75-79] Consequently, therapies aimed at reducing or eliminating activation of the NFkB pathway due to LPS stimulation are at the forefront of scientific evaluation.

Therapeutic agents

While there are different therapeutic approaches to endotoxemia, success comes from rapidly determining the underlying cause and subsequent implementation of appropriate therapy. One approach is to prevent translocation of endotoxin into circulation by removing affected structures such as devitalized bowel or an infected umbilicus. Another therapeutic approach is neutralization of the LPS molecule prior to its interaction with the effector cell population. Antiendotoxin antibodies targeted against the O-antigen of the LPS molecule is one therapeutic option.[2,3,29,80-84] These antibodies are collected from horses vaccinated against the core regions of the Re *Salmonella* mutant of the rough strains of J5 *Escherichia coli*.[2,3,29,53] However, these antibodies are specific for a bacterial strain and therefore do not afford much crossover protection. Additionally, antiendotoxin antibodies have shown conflicting results. Studies have reported

improvement of clinical signs in treated adult horses while others report no improvement or worsening of clinical signs in foals.[22,29,83,85] The exact reason for this discrepancy is not fully understood, however there are some explanations. It is possible the antibodies were administered too late in the disease process given that the interaction between endotoxin and equine inflammatory cells occurs rapidly.[3,22] Additionally, the antiendotoxin titer may be insufficient to effectively neutralize the endotoxin challenge.[3,22] Since the toxic moiety of the LPS molecule is the inner lipid A portion, the O-antigen antibodies may not penetrate the molecule to bind lipid A.[3,22] Therefore, the structurally invariant and biologically active center of LPS, lipid A, is a logical therapeutic target for neutralization (Fig. 1.3).

Lipid A is composed of a hydrophilic, negatively charged bisphosphorylated diglucosamine backbone, and a hydrophobic domain of 6 (*E. coli*) or 7 (*Salmonella*) acyl chains in amide and ester linkages (**Fig. 1.3**).[35,86-88] The anionic, amphiphilic nature of lipid A enables it to interact with a variety of cationic hydrophobic ligands.[89] Lipid A receptor antagonists such as the unusual lipid A derived from *Rhodobacter sphaeroides* or Polymyxin B (PMB) are strategies that have produced mixed results. Diphosphoryl lipid A from *R. sphaeroides* (RsDPLA) and the TLR4 antagonist E5531 inhibited binding of enteric LPS, cytokine release, and activity of LPS induced gene expression in human and murine models.[90,91] However, in horses, RsDPLA and E5531 behave as potent agonists.[90,91] This receptor-ligand interaction is suggested to result from the composition of equine TLR4.[91]

Polymyxin B is a cationic amphiphilic cyclic decapeptide antibiotic isolated from *Bacillus polymyxa* that has long been recognized to bind lipid A and neutralize its toxicity

in vitro in animal models of endotoxemia.[2,3,20,22,23,92,93] While not used in humans at standard antimicrobial dosages due to life-threatening toxicity, low dose PMB is a recognized beneficial form of therapy for endotoxemia in horses and foals.[2,3,22,23,53,92] Unlike *S. typhimurium* antiserum, polymyxin B exhibits beneficial effects when administered to endotoxemic foals by reducing TNF and IL-6 production.[22] However, studies have shown polymyxin B to exhibit toxic side effects such as ataxia when given intravenously every 6 hours at high dosages such as 36000 IU/kg in horses.[93] At the University of Georgia's Veterinary Medical Teaching Hospital, use of polymyxin B at a dose of 1000-5000 IU/kg given intravenously every 12 hours for 3 days duration has not demonstrated toxic side effects.[20] Given the potential for toxicity, it is recommended that polymyxin B be used judiciously in patients with renal compromise.

Lipopolysaccharide effector cell interaction initiates a cascade that culminates in the production of inflammatory mediators associated with the clinical signs of endotoxemia. In order to reduce morbidity, antiendotoxic therapy is targeted at prevention or reduction of synthesis, release or action of the inflammatory mediators.[2,3] Therapeutic efficacy of nonsteroidal anti-inflammatory drugs, specifically flunixin meglumine, results from prevention of prostaglandin synthesis and thromboxane while reducing clinical signs associated with endotoxemia.[2,3,94] Another option is administration of omega-3 fatty acids (alpha-linolenic and eicosapentaenoic acid).[3,27,95-97] The theory behind this strategy is to reduce the synthesis of proinflammatory cyclooxygenase-derived metabolites of arachidonic acid by replacing it with an alternative fatty acid. Different studies have evaluated the most efficacious route for administration. Intravenous administration of omega-3 fatty acid in a 20% lipid emulsion altered the fatty acid composition of

monocytes within 8 hours.[96] These changes persisted for roughly 1 week and the production of thromboxane and TNF were reduced.[96] Pentoxifylline is a rheologic agent used in humans that has demonstrated the ability to reduce production of cytokines and thromboxane.[18,19,94,98] While the beneficial effects of pentoxifylline alone are limited[19], when combined with flunixin meglumine the protective hemodynamic effects were greatly enhanced.[94]

Additionally, general supportive care strategies with intravenous fluids such as crystalloids and colloids are commonly used.[2] Currently, investigations into therapeutic agents that would prevent or interfere with endotoxin induced cellular activation are being explored. Given that no treatment options work completely and some therapeutics are toxic, novel therapeutic options are constantly being explored.

Recently, small molecule analogs of PMB have been under development. A small-molecule that has the binding ability of polymyxin B without the associated nephrotoxicity would be ideal. It was determined that the pharmacophore necessary for optimal recognition and neutralization of lipid A by small molecules requires two protonatable positive charges so disposed that the distance between them are equivalent to the distance between the two anionic phosphates on lipid A (~ 14 Å; see Fig. 1.3). This compound would have the ability to form ionic H-bonds between the phosphates on the lipid A backbone and the positive charges on the compound.[36] In addition, appropriately-positioned pendant hydrophobic functionalities are necessary to further enhance binding affinity and stabilize the resultant complexes via hydrophobic interactions with the polyacyl domain of lipid A (for a recent review, see David et al.[89]). These structural requisites were first identified in certain members of a novel class of

compounds, the lipopolyamines, which were originally developed, and are currently being used as DNA transfection (lipofection) reagents.[99-102] The compounds of the conjugated spermine class are of particular interest because they are active *in vitro* and afford protection in animal models of Gram-negative sepsis, are synthetically easily accessible, and are nontoxic due to their degradation to physiological substituents (spermine and fatty acid).[103,104]

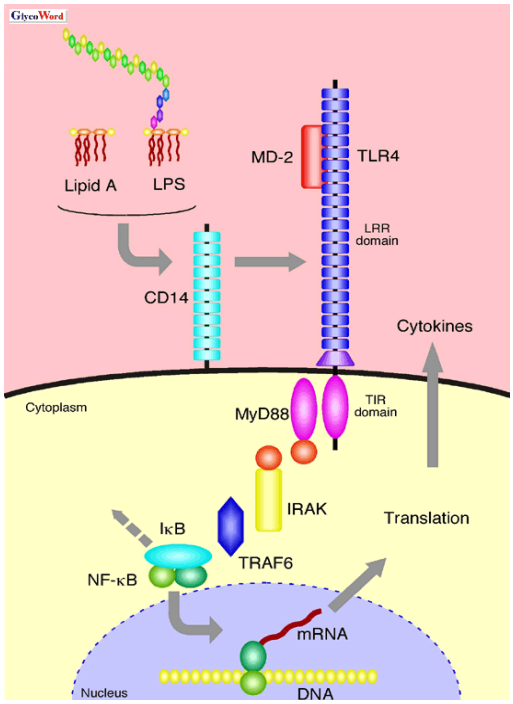
Preliminary Data:

Recent reports describe the synthesis and structure-activity relationships pertaining to the anti-endotoxic activities of several mono- and *bis*-acylated homologated spermine molecules.[36] Of these, EVK063 (**4e**[36]) was found to be as active as PMB in a panel of *in vitro* assays, including inhibition of proinflammatory cytokine production in LPS-stimulated human blood *ex vivo* (**Fig. 1.4**). In this study, EVK063 afforded complete protection against LPS-induced lethality in a murine model of shock, and yet, did not display any signs of demonstrable toxicity.[36] For the sake of brevity, only a summary of the *in vivo* data obtained in the murine model of shock is given below. The protective effects of **4e**, the most potent compound in the human TNF inhibition assay have been characterized using a well-established animal model of LPS-induced lethality in mice sensitized by D-galactosamine.[78,79] A supralethal dose (twice the dose causing 100% lethality) of endotoxin (200 ng/mouse) was administered intraperitoneally (i.p.) to groups of 5 mice sensitized with D-galactosamine. Experimental treatments consisted of concurrent, separate intraperitoneal injections of graded doses of compound, and lethality was observed at 24 h. As is evident from **Table 1.1**, a clear dose-response is observed, with **4e** affording complete (statistically significant) protection at the 100 or

200μg/mouse dose, and partial protection at the 50μg dose. In earlier studies on DOSPER, the first lipopolyamines PMB mimic that was studied,[104] it was observed that the temporal window of protection was very short. DOSPER had to be administered concurrent with the LPS challenge, and significant mortality resulted when the compound was given even 15 minutes prior to LPS. This was attributed to the extreme hydrolytic susceptibility to serum esterases of the *ortho*-ester linkages of the oleoyl groups,[104] and it was of interest if the amide-linked acylhomospermines would display a longer plasma half-life, thus affording a longer time-window of protection. It is evident from the time-course experiment (**Table 1.2**) that **4e** is indeed apparently much longer-lived, with near-complete protection evident when the compound is administered 6 h prior to LPS challenge. In a few cohorts, the observed time window of protection was 4 h when **4e** was administered intravenously (i.v.), and was 8h when given subcutaneously, suggesting a depot effect and prolonged release into systemic circulation in the latter experiments.[36]

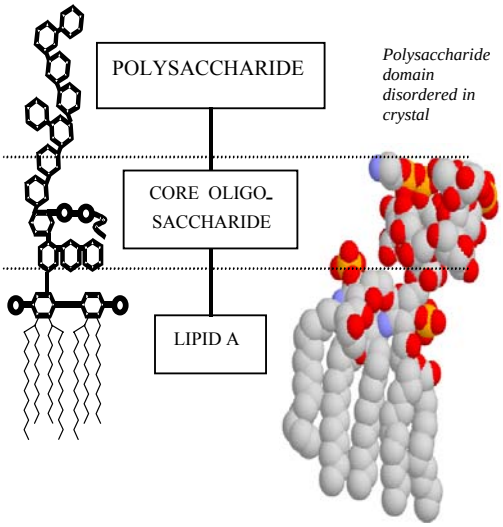
Although investigations testing the utility of EVK063 have shown promising results in rodent models, it has not yet been tested in a large animal model. As mentioned earlier, horses are exquisitely sensitive to the effects of endotoxin and the clinical signs of endotoxemia have been well documented. While not possible in rodent models, parametric data invaluable to enhancing our understanding of the endotoxemic cascade can be easily obtained in the equine, making the horse an appropriate model to study.

Fig. 1.1. Schematic of LPS signaling pathway



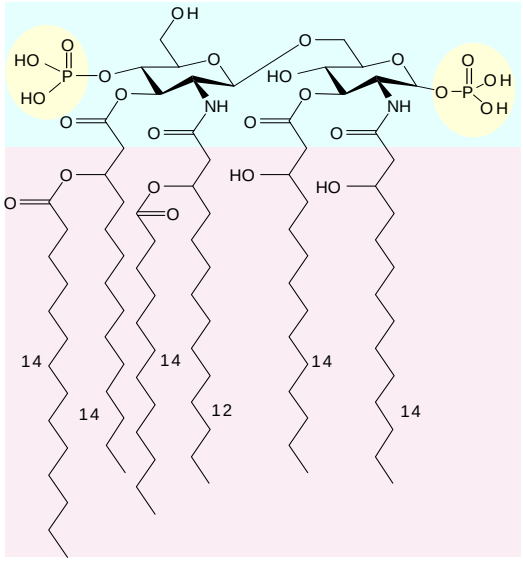
http://www.glycoforum.gr.jp/science/word/immunity/IS-A01_images/fig03.gif

Fig. 1.2 Schematic (*left*), and crystal structure (*right*) of lipopolysaccharide (LPS). Atoms are shown colored in standard CPK scheme.



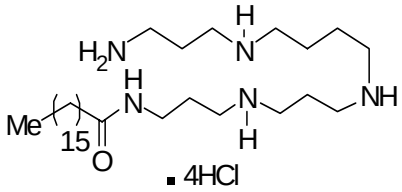
Courtesy of Sunil David, MD, PhD; Lawrence, KS

Fig. 1.3. **Structure of Lipid A.** The presence of anionic, hydrophilic, and hydrophobic domains enable the binding of cationic amphipaths to lipid A.



Courtesy of Sunil David, MD, PhD; Lawrence, KS

Fig. 1.4. Structure of EVK063 (**4e**)



Courtesy of Sunil David, MD, PhD; Lawrence, KS

Table 1.1. Dose-dependent protection of CF-1 mice challenged with a supralethal dose of 200 ng/mouse (LD_{100} = 100 ng) by EVK063/**4e** in cohorts of five animals. Lethality was recorded at 24 h post-challenge. Ratios denote dead/total. Asterixes indicate statistically significant values, $p<0.05$.

Dose (μg/mouse)	Compound 4e
200	0/5*
100	0/5*
50	2/5
10	5/5
0	5/5

Table 1.2. Time-course of protection afforded by EVK063/**4e** in the D-galactosamine sensitized CF-1 mouse lethality model. Animals were injected with 200 μg 4e i.p. at times noted with respect to LPS challenge (200 ng/mouse). Lethality was recorded at 24 h following LPS challenge. Asterixes indicate statistically significant values, $p<0.05$.

Time of 4e Administration	Lethality (dead/total)
-6h	1/5*
-4h	1/5*
-2h	0/5*
0h	0/5*
+1h	5/5
+2h	4/5

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Chapter 2

Introduction

Despite constant medical advancements, endotoxemia remains a leading cause of death in the horse.[1-5] Endotoxins, or lipopolysaccharides (LPS), are liberated from the outer leaflet of the cell wall outer membrane during either rapid proliferation or lysis of a Gram-negative microorganism.[6] Since fermentation occurs in the hind gut of the horse, the gastrointestinal tract naturally contains Gram-negative bacteria and large amounts of LPS.[7] The gastrointestinal epithelium provides a barrier to prevent LPS absorption; however, when epithelial disruption occurs, as in some colic (mechanical disruption) and colitis (inflammatory disruption) cases, clinical consequences can be devastating. A similar scenario is observed in humans where gram-negative sepsis remains a leading cause of overall mortality and the number one non-coronary cause of death in the intensive care unit.[8] Human hospital costs associated with the treatment of endotoxemia exceed 17 billion dollars annually and continue to rise.[8] Considering the enormous economic burden and high mortality rates, an animal model with recognized sensitivity to LPS would be invaluable in evaluation of novel therapeutics.

Lipopolysaccharide consists of a polysaccharide portion and a lipid called lipid A (Fig. 1.2). The polysaccharide portion consists of a polymer of repeating oligosaccharide units, the composition of which is highly varied among gram-negative bacteria. A relatively well-conserved core hetero-oligosaccharide covalently bridges the polysaccharide with lipid A.[9,10] The structurally highly conserved lipid A is the active moiety of LPS.[11-13]

Our understanding of basic mechanisms underlying the cellular response to LPS has increased in recent years. Research contributions demonstrate LPS recognition by CD14[14-23] via an LPS-binding acute-phase plasma protein (LBP),[14,23-31] followed by initiation of signal transduction by Toll-like receptor-4[32-42] and Myeloid Differentiation Factor 2.[32,43-47] These interactions allow translocation of LPS into the cytoplasm where cellular activation events lead to nuclear translocation of Nuclear Factor kappa B (NF-κB)[15,48-51] resulting in cytokine mRNA transcription. Tumor Necrosis Factor-α (TNF) and Interleukin-6 (IL6) are two predominant inflammatory mediators released subsequent to activation of the NF-κB pathway,[5,25,49-58] and are mainly secreted from the macrophage, the primary effector host cell associated with sepsis.[20,28,58-60] Consequently, studies have shown both TNF and IL6 are elevated during naturally occurring, as well as iatrogenically-induced, endotoxemia with TNF activity slightly preceding that of IL6.[5,53,55,56] The up-regulation of TNF expression correlates with pyrexia, tachycardia, lethargy and leukopenia, while IL6 correlates with up-regulation of acute phase proteins, cellular differentiation and pyrexia.[3,5,55,61]

Therapeutic strategies attempt to bind and sequester LPS prior to its interaction with its effector cell. The most recognized method involves binding Lipid A, the toxic portion of the LPS molecule, to mask the binding region in an attempt to decrease the inflammatory response. While several options are available, none are completely effective. Anti-endotoxin antibodies[60,62-69], pentoxifylline[50,62,63,70-72], polymyxin B (PMB)[1,2,4,60,62,63,73,74], antibiotics and non-steroidal anti-inflammatory drugs (NSAIDs)[60,62,63,70,75] have minimal efficacy. Additionally, each therapeutic agent does not elicit the same response in all species; with many having negative side effects.

Recently, a novel molecule, EVK063, has been developed. EVK063 is a C_{16} monoacyl homospermine compound with comparable binding strength and potency to polymyxin B.[76] Unlike polymyxin B, EVK063 is broken down into the metabolically non-toxic molecules spermine and fatty acid and therefore does not exhibit the associated oto-, neuro- and nephrotoxicity.[76] Although investigations testing the utility of EVK063 have shown promising results in rodent models, it has yet to be tested in a large animal model.

The horse is exquisitely susceptible to the effects of endotoxin and is commonly affected with the clinical syndrome of endotoxemia, resulting in death. Moreover, this inherent sensitivity makes the horse an appropriate model for testing anti-endotoxin therapies. Therefore, the purpose of this study was to assess the ability of EVK063 to inhibit the inflammatory response of LPS-challenged equine peripheral blood mononuclear cells (PBMCs) *in vitro*. We hypothesize that EVK063 will inhibit cytokine production by endotoxin-challenged equine PBMCs in a manner comparable to PMB.

Method Development

Endotoxin is present throughout the environment. Consequently, horses are naturally exposed to various amounts and types of endotoxin throughout their lives, creating biological variability. Therefore, in order to appropriately characterize the effects of EVK063 on the host cell, our experimental protocol was carried out in series. The first stage consisted of incubating samples in culture media without autologous serum. This allowed cell-EVK063 interaction to be determined without the influence of proteins or other molecules. The second stage consisted of incubating samples in culture media that contained 10% autologous serum. We chose 10% serum to stay consistent with previous work.[77,78] This permitted evaluation of potential protein interactions that have been

observed in previous investigations of EVK063. The third stage consisted of incubating samples in whole blood to allow assessment of additional interactions not observed in the previous two stages.

Materials and Methods

First stage – with and without serum: Whole blood was collected from eight healthy horses in pre-heparinized syringes using an approved institutional animal care and use committee protocol. Blood was then placed into 50mL conical screw top tubes and allowed to settle for 30 minutes. The plasma was layered over Ficoll® 1.077[a] (radiopaque polysucrose) in a 60/40 plasma/Ficoll ratio and centrifuged for 30 minutes at 400 X g. The mononuclear rich middle layer was removed, placed in a separate 50mL conical screw top tubes and centrifuged for 10 minutes at 400 X g. The supernatant was decanted and the pellet consisting of mononuclear cells was washed with phosphate buffered saline. The pellet was suspended in 1640 RPMI media[b] and replicate aliquots (concentrated at 4-5 million cells/mL) were placed in 6 well culture plates containing 1640 RPMI culture media without serum. The same treatments were assigned to samples incubated with and without serum as designated in **Table 2.1**. Samples to be stimulated with *Salmonella typhimurium* LPS[c] (100ng/mL)[79] were treated with graded concentrations of EVK063, [0.01μM (E0.01), 0.1μM (E0.1), 1μM (E1), 10μM (E10)], and 10μM PMB[d] (PL), then incubated at 37°C, 5%CO_2 for 6 hours. Non LPS stimulated samples consisted of a control with no treatment (C), vehicle control with 10uL Dimethyl Sulfoxide (DMSO)[e] (VC), 10μM PMB control (P) and 10μM EVK063 control (EC). One sample was stimulated with LPS but received no treatment (L). All wells had a final volume of 4mL. Media samples were collected and stored at -80°C for TNF protein

analysis while RNA was harvested using a commercial kit (Qiagen, Valencia, CA) and stored at -80°C for IL6 gene expression analysis. Following analysis, samples were incubated in culture media that contained 10% autologous serum. Treatments were assigned to samples as designated in **Table 2.1**. Following analysis of the intial experiment, the study was replicated with samples incubated with 10% autologous serum, a whole blood model was developed.

Second stage – whole blood: Whole blood was collected from each horse via jugular veinapuncture into a syringe containing 50µL heparin per 1mL blood. Nine milliliters of heparanized blood was placed into each of 6 T-75 culture flask's containing 9mL of 1640 RPMI media. Treatments were assigned as designated in **Table 2.2**.

Non LPS stimulated samples consisted of a control with no treatment (C), 10µM PMB control (P) and 10µM EVK063 control (EC). Three samples were stimulated with 2ng/mL *Salmonella typhimurium* LPS. One sample received no treatment (L), one received 10µM PMB (PL) and one received 10µM EVK063 (EL). Flask's were placed on an agitator and incubated at 37ºC, 5% CO2 for 6 hours. After incubation, the diluted whole blood was removed and layered over 20 mL of Ficoll 1.077 in a 50 mL conical screw top tube and centrifuged for 30 minutes at 400 X g. The mononuclear rich middle layer was removed and placed in a separate 15mL conical screw top tube and centrifuged for 10 minutes at 400 X g. The supernatant was decanted and frozen at -80ºC for TNF evaluation. The cell pellet was washed with Phosphate Buffered Saline (PBS) and subsequently disrupted to allow collection of 200µL for flow cytometry. The remaining cell suspension was centrifuged for 10 minutes at 400 X g to create a cell pellet. Ribonucleic acid (RNA) was harvested using a commercial kit (Qiagen, Valencia, CA).

RNA Isolation

The cell pellet was disrupted using Buffer RLT Plus. The solution was then transferred to a QIAshredder spin column and centrifuged for 2 minutes at 16.1g. The homogenized lysate was transferred to a gDNA Eliminator spin column to which an equal volume of 70% ETOH was added and centrifuged at 16.1g for 30sec. The samples were washed once with RW1 Buffer and twice with RPE Buffer and centrifuged following instructions. RNase-free water was added to the spin column and centrifuged at 16.1g for 1min, allowing collection of the RNA into an RNase-free collection tube. Storage solution (RNA) was added in equal volume to each sample. The samples were aliquoted in 10µL increments and frozen at -80ºC. NanoDrop confirmed RNA quantity and IL6 gene expression was analyzed using qRT-PCR (Invitrogen, Carlsbad, CA) with primer sets specific for equine IL6 and 18s (**Table 2.3**).

Real Time quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

The SuperScript III Platinum One-Step Quantitative RT-PCR system (Invitrogen, Carlsbad, CA) in combination with sequence specific primers and a labeled fluorogenic probe was employed for the analysis of IL6 message. Analysis of the housekeeping gene 18s was performed in the presence of 0.2x SYBR green I (Molecular Probes, Eugene, OR) with primers obtained from a commercially available equine kit (TaqMan, ribosomal RNA, Applied Biosystems, Foster City, CA). All qRT-PCR reactions were run on a Cepheid SmartCycler (Sunnyvale, CA) with RNA from treated cells. Thermocycling protocols for IL6 were as follows: Stage 1; 50.0°C for 120 seconds, 95.0°C for 600 seconds; Stage 2; 40 cycles of 95.0°C for 15 seconds, 63.0°C for 30 seconds, 72.0°C for

30 seconds, 78.0°C for 7 seconds, 78.0°C for 13 seconds. Melt Curves were performed at 60.0°C to 95.0°C at 0.2°C/second. Gel electrophoresis in the presence of 2% ethidium bromide confirmed that IL6 was produced at 214 base pairs.

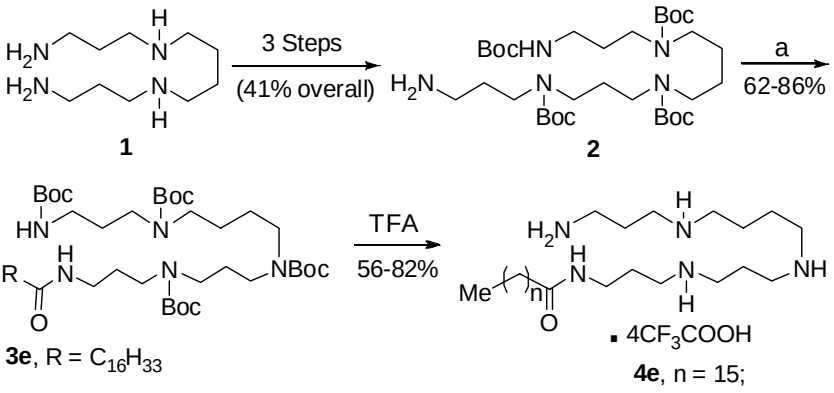
Tumor Necrosis Factor Quantification

Quantification of TNF was by an equine specific ELISA kit (Endogen®, Rockford, IL). The technique will be described with incubation occurring at room temperature unless otherwise stated. Briefly, 100µL of coating antibody in carbonate/bicarbonate buffer solution was placed in each well and incubated overnight at 4ºC. Blocking buffer was then added to each well for 1 hour. After a 3 wash cycle with PBS'T, the appropriate dilution of standard and samples were then added to each well and incubated for 90 minutes. The wells were then washed 3 times with PBS'T and 100µL of detection antibody solution added to each well. Following 60-minute incubation and a wash cycle, 100µL of streptavidin-HRP was added and the wells incubated for 30 minutes. Another wash cycle was performed and 100µL of substrate placed in each well. The plate was incubated in the dark for 20 minutes. A stop solution of H_2SO_4 was added prior to reading on a micro-elisa plate reader.

Flow Cytometry

Cellular viability was determined using propidium iodide staining (PI, 2 mg ml^{-1}) a dye that intercalates with nucleic acids to determine if an interruption in the cell membrane existed following damage or cell death. Duplicate 200µL aliquots from each sample were reserved for analysis of cellular viability. One aliquot served as control and the second aliquot received 10µL of Propidium Iodide. Samples were run on the FACS Calibur (Becton Dickinson Immunocytometry system serial # E4400).

Synthesis of EVK063: The compound was synthesized using published procedures[76] as summarized in Scheme 1.



Scheme 1. Reagents: (a) RCOOH, EDCI, THF, 10 h

Statistics

Statistical evaluation was performed using Analysis of Variance with significance set at $p \leq 0.05$. Using the mean squares for treatment and error, the F statistic allowed acceptance or rejection of the research hypothesis. Multiple pairwise comparisons of treatment means were made using the Tukey method. The experimental error rate was set at 0.05. All computations were performed using Statistical Analysis Software (SAS® Cary, NC). The data was then normalized to the control sample (100% inhibition) and the LPS sample (0% inhibition) for presentation.

Results

Samples incubated without serum

Lipopolysaccharide stimulated equine PBMC's were incubated without serum and evaluated for TNF production and IL6 expression. All samples were treated under the

same conditions to minimize variability in technique. Tumor necrosis factor ELISA analysis revealed a concentration dependent reduction in TNF production (**Fig 2.1, Table 2.4**). Non LPS stimulated samples (C, VC, P, EC) demonstrated significantly lower TNF production when compared to the non-treated LPS stimulated sample (L), but did not show significance between each other. Samples stimulated with LPS and treated with 0.01μM, 0.1μM and 1μM concentrations of EVK063 were unable to reduce TNF production by more that 27% when compared to the untreated LPS stimulated sample. The 10μM EVK063 concentration inhibited TNF production (84-98%), which was consistent with the LPS stimulated 10μM PMB treated sample.

Interleukin-6 analysis by qRT-PCR was performed in the same manner as described for TNF analysis. Samples were normalized to control and fold increase in IL6 gene expression was determined (**Fig 2.2, Table 2.4**). Non LPS stimulated samples (C, VC, P, EC) showed statistical significance in IL6 expression when compared to the untreated LPS stimulated sample, but did not show significance between each other. At 0.01μM, 0.1μM, and 1μM concentrations there was no effect of EVK063 on IL6 expression in LPS treated cells. The 10μM EVK063 significantly reduced IL6 expression (67-82%), similar to the effect of 10μM PMB treatment.

Flow Cytometry of samples incubated without serum

Cellular viability between 94-97% was maintained in the following samples: C, VC, L, E0.01, E0.1, P and PL. However, the E1 sample exhibited 15-25% cell death while the EC and E10 samples exhibited 62-72% cell death.

Samples incubated with 10% Autologous serum

Equine PBMCs were evaluated in culture media that contained 10% autologous serum using the same 10 treatment conditions as previously developed (**Fig. 2.3, Table2.5**). Non LPS stimulated samples (C, VC, P, EC) demonstrated significantly lower TNF production when compared to the untreated LPS stimulated sample, but did not show significance between each other. Inhibition of TNF at 0.01μM, 0.1μM and 1μM EVK063 concentrations ranged from 5-37%. The 10μM EVK063 sample inhibited TNF production (86-97%), which was comparable to the LPS stimulated 10μM PMB treated sample.

Interleukin-6 analysis by qRT-PCR was performed with 10% autologous serum as described for TNF analysis. Samples were normalized to control and fold increase in IL6 gene expression was determined (**Fig. 2.4, Table 2.5**). Non LPS stimulated samples (C, VC, P, EC) demonstrated statistical significance in IL6 expression when compared to the untreated LPS stimulated sample but did not show significance between each other. The 0.01μM, 0.1μM, and 1μM EVK063 concentrations showed minimal reduction in IL6 expression when compared to the untreated LPS stimulated sample. Interleukin-6 expression was reduced (71-84%) in the LPS stimulated 10μM EVK063 sample, which was comparable to the LPS stimulated 10μM PMB treated sample.

Flow Cytometry of samples incubated with 10% Autologous serum

Cellular viability between 92-97% was maintained in the following samples: C, VC, L, E0.01, E0.1, E1, P and PL. EC and E10 samples exhibited 35-39% cell death.

Samples incubated with whole blood

The following samples were used in the whole blood model: C, L, P, PL, EC, E10. The 10μM EVK063 concentration inhibited tumor necrosis factor production (73-81%) (**Fig 2.5**) and IL-6 gene expression (69-75%) (**Fig 2.6**) at levels similar to the 10μM PMB sample.

Flow Cytometry of Samples incubated with whole blood

All samples maintained 92-98% cellular viability.

Discussion

Data from this investigation demonstrates the ability of EVK063 to reduce TNF production and IL6 expression by LPS stimulated mononuclear cells *in vitro* with comparable results to PMB. EVK063 is a novel lipopolysaccharide binding pharmacophore with the ability to attenuate the effects of endotoxemia in lab animal models with comparable results to PMB. We chose to evaluate TNF and IL6 due to their documented role in endotoxemia and to remain consistent with previous work regarding evaluation of PMB.[1,2,4,52,73]

We examined EVK063 at concentrations of 0.01μM, 0.1μM, 1μM and 10μM. A 10μM PMB concentration served as the standard for inhibition of cytokine synthesis. Previous investigations have demonstrated the ability of EVK063 to bind LPS in a similar manner to PMB. [80] Therefore, we chose 10μM PMB to stay consistent with previous work and to obtain the same concentration as the highest sample of EVK063.[80]

Originally all 8 samples were incubated in culture media without autologous serum. This allowed evaluation of the EVK063-cell interaction without interference from protein or other plasma constituents. While the 10μM sample demonstrated inhibition of TNF

production and IL6 expression comparable to PMB, the lower concentrations of EVK063 did not exhibit greater than 30% inhibition. However, flow cytometry determined the samples containing 10μM EVK063 experienced 70% cell death and the 1μM EVK063 sample experienced 25% cell death. All other treatments groups experienced less than 7% cell death. We were concerned the higher concentrations of EVK063 created an unsuitable osmolar environment that may have lead to cell lysis, however, media from all samples were evaluated with an osmometer and determined to be within normal range (300-330mOsm). Given the amount of cell death, confirmation as to the efficacy of EVK063 at 1μM and 10μM concentrations could not be made. However, we concluded that the treatment groups containing 0.01μM and 0.1μM EVK063 were ineffective at inhibiting TNF production and IL6 expression under 0% serum conditions.

We then evaluated all sample conditions in culture media with 10% autologous serum to determine if the toxicity at 1μM and 10μM concentrations of EVK063 could be attenuated, or the efficacy of the 0.01μM and 0.1μM concentrations of EVK063 enhanced.

Tumor necrosis factor and IL6 inhibition results with 10% autologous serum conditions were similar to our first trials without serum. We were able to determine that the 0.01μM, 0.1μM and 1μM EVK063 concentrations were ineffective at inhibiting TNF production and IL6 expression in LPS stimulated mononuclear cells under 10% serum conditions. However, cell death was reduced from 70% to 35%, for without and with serum respectively, in the 10μM EVK063 samples and 8% in the 1uM concentration as confirmed by flow cytometry. Even though cell death was reduced to 35% for 10μM concentrations, we considered this value unacceptable and an interpretation could not be

made about the efficacy of the 10μM EVK06 concentration. Following these results we evaluated the 10μM concentration of EVK063 in a whole blood model.

We used previous whole blood and *ex vivo* models evaluating PMB to determine the amount of LPS used for stimulation.[1,3,4,61,79] Evaluation of whole blood samples again showed a reduction in TNF production and IL6 expression when compared to non-treated LPS stimulated samples. Evaluation of samples via flow cytometry demonstrated no difference in cell death between control and treated samples. Therefore, the results from the whole blood model confirmed the ability of EVK063 at a 10μM concentration to attenuate cytokine production from LPS stimulated mononuclear cells with comparable results to 10μM PMB.

The improvement in cell viability after adding autologous serum to the culture media was not unexpected. Previous work in murine models demonstrated attenuation of toxicity when the compound was administered in conjunction with albumin.[76] These findings indicate a fraction of the compound binds to albumin and thus reduces toxicity.[76] The mechanism of this attenuation is unclear at this time.[76] However many endotoxic patients are hypoproteinemic, therefore, further investigations should evaluate the amount of protein binding by EVK063.

A shortcoming of this study is the low number of samples in the whole blood model. While we concluded the ability of a 10μM concentration of EVK063 to inhibit TNF production and IL6 expression, additional samples are required to increase the power and validity of these findings. Additionally, we only evaluated a 10μM concentration of EVK063 in the whole blood model. Therefore, while we concluded that the 0.01μM, 0.1μM and 1μM EVK063 concentrations were ineffective at inhibiting TNF production

and IL6 expression under 0% and 10% serum conditions; these concentrations should be evaluated in a whole blood model.

Additional whole blood investigations should also evaluate the potential for synergistic effects with known therapeutic options, such as Polymyxin B. Polymyxin B is a cationic, amphiphilic cyclic decapeptide antibiotic that has long been recognized to bind lipid A and neutralize its toxicity in animal models of endotoxemia both *in vitro* and *in vivo*.[1,2,4,62,63,73,74,81] However, side effects of Polymyxin B including neurotoxicity and nephrotoxicity are well documented. Therefore, Polymyxin B is a recognized beneficial form of therapy in endotoxemic horses and foals, but must be used judiciously.[2,4,60,62,63,73] In order to reduce the toxic effects of PMB, investigations have evaluated conjugating PMB with additional molecules such as dextran-70.[2,82,83] Dextran-70 was chosen due to its use as a plasma expander and its molecular weight limits the loss of PMB from the intravascular space.[82] The conjugated molecule of Polymyxin B-Dextran-70 (PMB-70) has demonstrated the ability to neutralize endotoxin in a murine, equine and ovine model of endotoxemia.[2,82,83] Additionally, neither neurotoxocity or nephrotoxicity were observed in the equine model.[2] However, specie specificity often precludes the use of novel therapeutics.

Species specificity has made the development of novel antiendotoxic therapeutics challenging. Antiendotoxic therapeutics such as *Rhodobacter sphaeroides* and E5531 have shown promising results in lab animal models yet have failed to demonstrated beneficial effects in the equine model.[84,85] In some cases they acted as an agonist after LPS stimulation in equine mononuclear cells.[84,85] One explanation for these differences is structural modification of the molecule within the equine model.[84] While there are no

data to support the theory, it was suggested by the authors that neutrophils may release enzymes that could alter the biological activity of the compound.[84]

Even with constant advancements in therapeutics, endotoxemia remains a devastating clinical problem for both human and veterinary practitioners.

While fewer than 100 cases were documented prior to 1920, sepsis is now recognized to be one of the leading causes of mortality and the number one non-coronary cause of death in the human intensive care unit, accounting for greater than 215,000 deaths a year.[86] While known therapeutics like PMB are recognized as short term treatment options for animal models, the life threatening toxicity in humans precludes its use. Therefore, a molecule with the ability to bind LPS with the same potency as PMB without exhibiting the toxic side effects would be ideal. This investigation demonstrates the ability of EVK063, a novel lipopolysaccharide binding pharmacophore, to inhibit cytokine synthesis from LPS stimulated equine mononuclear cells *in vitro*.

While much work is still required prior to *ex vivo* and *in vivo* assessment of EVK063, a positive groundwork has been established. This *in vitro* study confirms the ability of EVK063 to inhibit TNF production and IL6 expression in LPS stimulated equine mononuclear cells with comparable results to PMB.

[a]Ficoll® 1.077; Sigma-Aldrich®; St. Louis, MO
[b]HyQ® RPMI-1640 Medium (1x); Logan, UT
[c]Lipopolysaccharide from *S. typhimurium*; List Biological Laboratories; Campbell, CA
[d]Polymyxin B; Bedford Laboratories™; Bedford, OH
[e]Dimethyl Sulfoxide; Sigma-Aldrich®; St. Louis, MO

Figure Legend

Sample ID	
C	Control
VC	Vehicle Control (10 µL DMSO)
L	LPS
P	PMB (10 µM)
PL	PMB (10 µM) + LPS
EC	EVK063 10 µM
E10	EVK063 10 µM + LPS
E1	EVK063 1 µM + LPS
E0.1	EVK063 0.1 µM + LPS
E0.01	EVK063 0.01 µM + LPS

Figure 2.1: **Tumor Necrosis Factor inhibition: cells incubated without serum**

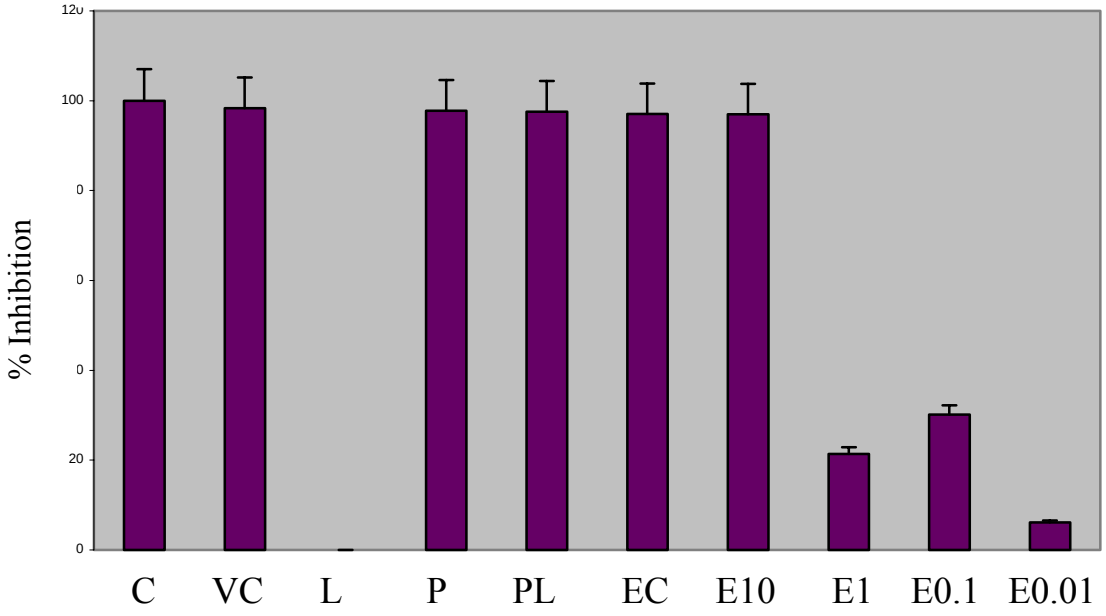


Figure 2.2: **Interleukin-6 inhibition: cells incubated without serum**

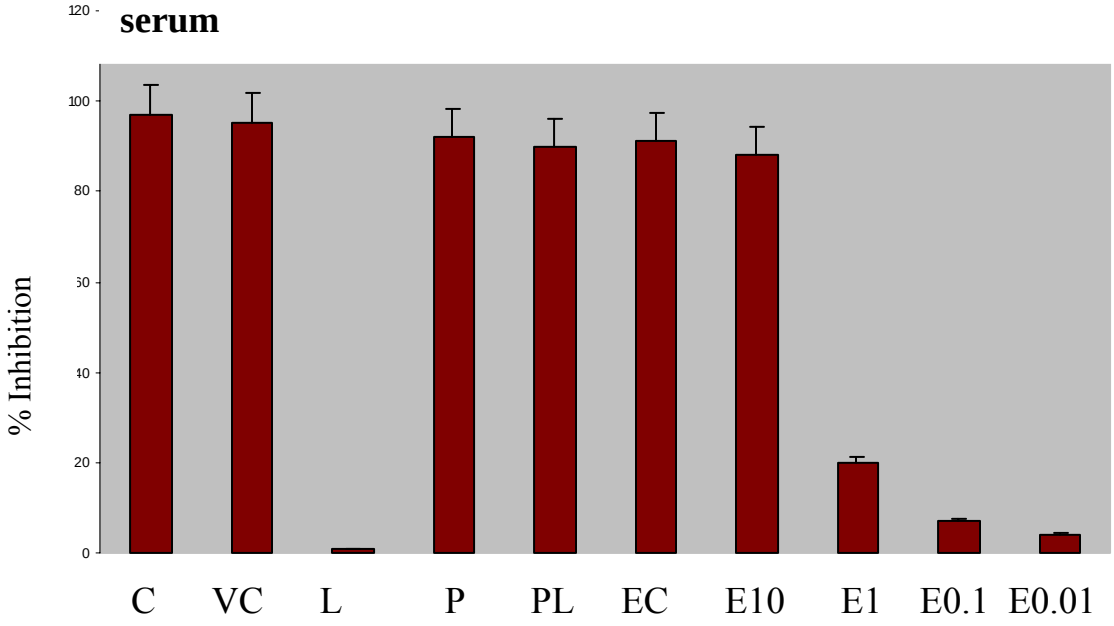


Figure 2.3: **Tumor Necrosis Factor inhibition: cells incubated with 10% autologous serum**

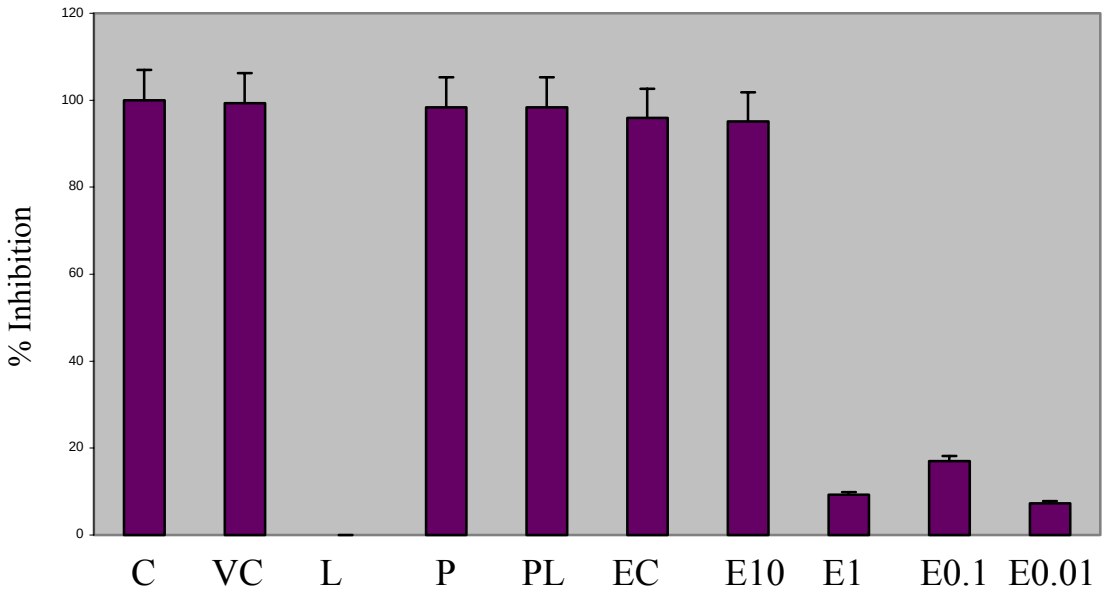


Figure 2.4: **Interleukin-6 inhibition: cells incubated with 10% autologous serum**

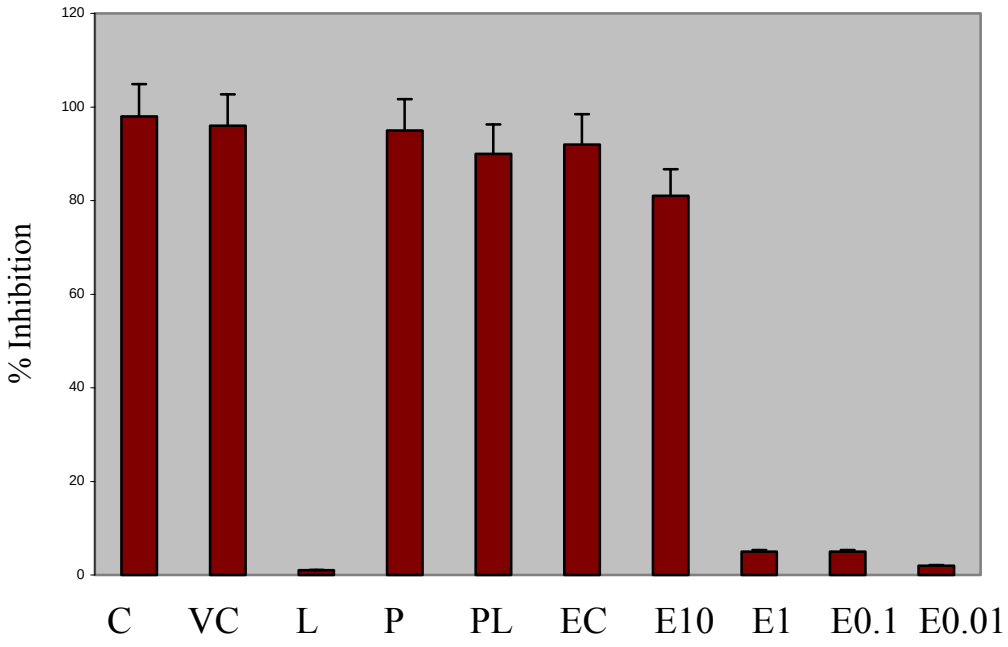


Figure 2.5: **Tumor Necrosis Factor inhibition: whole blood**

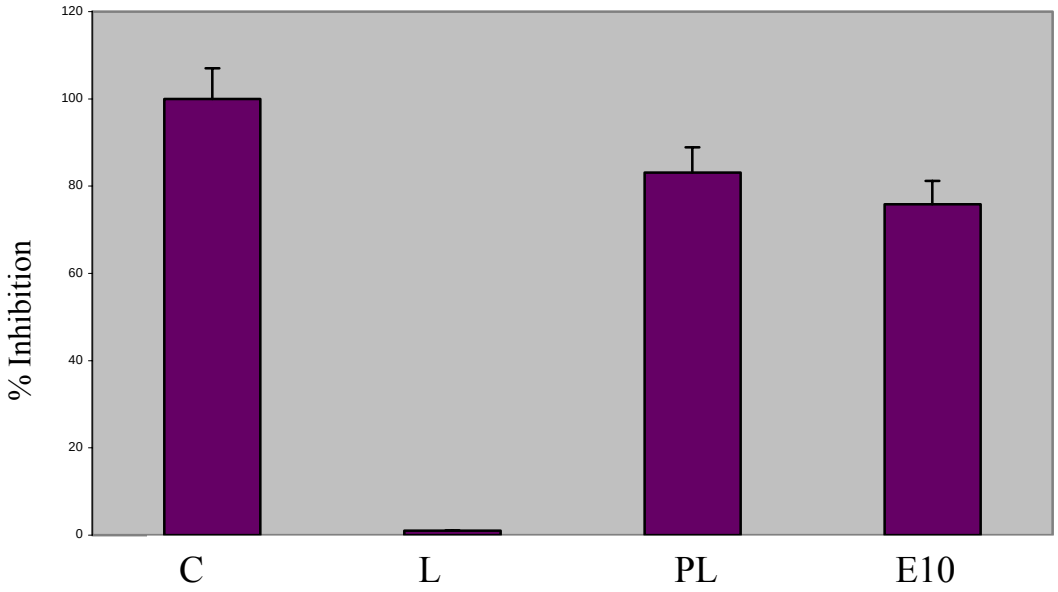


Figure 2.6: **Interleukin-6 inhibition: whole blood**

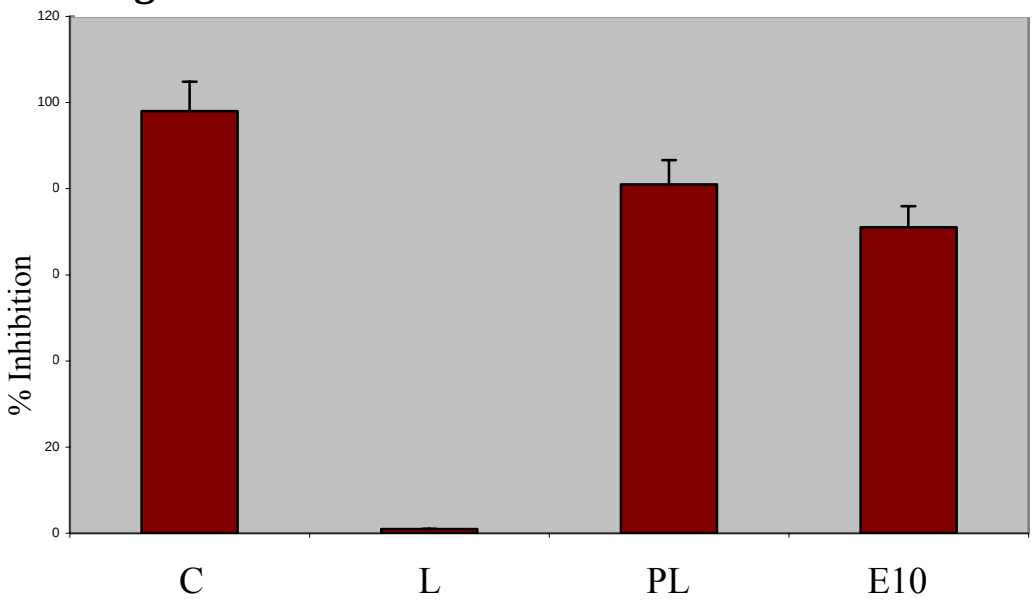


Table 2.1: Sample Identification: with and without 10% autologous serum

Sample ID	Treatment	*S. typhimurium* LPS
C	none	0
VC	Vehicle (10 µL DMSO)	0
P	PMB (10 µM)	0
EC	EVK063 (10 µM)	0
L	none	100ng/mL
PL	PMB 10 µM	100ng/mL
E10	EVK063 10 µM	100ng/mL
E1	EVK063 1 µM	100ng/mL
E0.1	EVK063 0.1 µM	100ng/mL
E0.01	EVK063 0.01 µM	100ng/mL

Table 2.2: Sample identification: whole blood model

Sample ID	Treatment	*S. typhimurium* LPS
C	None	0
L	None	2ng/mL
P	PMB 10µM	0
PL	PMB 10µM	2ng/mL
EC	EVK063 10µM	0
E10	EVK063 10µM	2ng/mL

Table 2.3: IL6 specific primers; product length: 214bp

Left primer	CCCCTGACCCAACTGCAA
Right primer	TGTGCCCAGTGGACAGGTTT
Hyb Oligo	CCTGCTGGCTAAGCTGCATTCACAG

Table 2.4: Tumor Necrosis Factor and Interleukin 6 inhibition: cells incubated without serum

Sample ID	Tumor Necrosis Factor % inhibition	Interleukin 6 % inhibition
C	100	100
VC	98	96
P	97	93
EC	95	91
L	0^{*+}	0^{*+}
PL	96	90
E10	95	88
E1	21^{*+}	20^{*+}
E0.1	30^{*+}	7^{*+}
E0.01	6^{*+}	4^{*+}

*comparison to PL with significance at $p \leq 0.05$
$^{+}$comparison to E10 with significance at $p \leq 0.05$

Table 2.5: Tumor Necrosis Factor and Interleukin 6 inhibition: cells incubated with 10% autologous serum

Sample ID	Tumor Necrosis Factor % inhibition	Interleukin 6 % inhibition
C	100	100
VC	99	95
P	98	95
EC	95	92
L	0^{*+}	0^{*+}
PL	97	90
E10	95	81
E1	9^{*+}	5^{*+}
E0.1	17^{*+}	5^{*+}
E0.01	7^{*+}	2^{*+}

*comparison to PL with significance at $p \leq 0.05$
$^{+}$comparison to E10 with significance at $p \leq 0.05$

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Chapter 3

Future Investigations

While positive findings were obtained regarding the ability of EVK063 to attenuate cytokine production from lipopolysaccharide (LPS) stimulated equine peripheral blood mononuclear cells (PBMC's), there is an imminent need for the continued exploration into the efficacy of EVK063. While Tumor necrosis factor-α (TNF) production and Interleukin-6 (IL6) expression were reduced when compared with non-treated LPS stimulated samples, their values were elevated compared to control samples. Therefore, further evaluation to determine the concentration of EVK063 that maximally inhibits cytokine production without displaying toxic effects on the cells *in vitro* would be prudent. Given the results from previous evaluations and this study, a whole blood model should be used for additional *in vitro* evaluations of EVK063.

We administered LPS and the assigned treatment to the cells at time 0. In previous investigations, EVK063 exhibited a time dependent relationship of protection from LPS stimulation in a murine model.[1] When administered as little as one hour post LPS challenge, all mice in the cohorts died after 24 hours.[1] While factors predisposing patients to endotoxemia are documented, most causes are not recognized prior to insult. In select cases, the source of endotoxin can be successfully removed. However, many times this is not possible; therefore, investigations evaluating the protective effects of EVK063 administered both pre and post LPS challenge are essential. This investigation focused on the cytokines TNF and IL6. Additional cytokines such as IL1, IL2 and IL6 should be evaluated to determine if EVK063 has the ability to attenuate their production.

An NFkB induction assay has been utilized in lab animal models.[2-4] and could provide valuable information if configured to evaluate equine samples. If EVK063 could inhibit activation of the NFkB pathway, several devastating clinical signs could be reduced. A p38 mitogen-activated kinase (MAPK) flow cytometric assay has also been utilized in lab animal models.[2-4] However, it was unclear if p38 MAPK existed in equine cells as it had not been described in the literature.[5] Recently, two separate studies have documented that p38 MAPK is essential in the equine LPS induction pathway.[6,7] Therefore, adaptation of the p38 MAPK flow cytometric assay to equine cells would prove beneficial.

Due to the severity of endotoxemia and the lack of appropriate therapies, multiple therapeutic agents are often used in conjunction. Therefore, after establishing the appropriate concentration of EVK063 for maximal inhibition, investigations should evaluate possible synergy with additional therapeutics. Non-steroidal anti-inflammatory drugs such as flunixin meglumine[5,8-11], antibiotics[5,8,9] and polymyxin B[5,8,9,12-16] are therapeutic agents that are commonly used in endotoxic patients. *In vitro* assessment using combinations of these agents with EVK063 should be considered.

Once sufficient *in vitro* data are obtained demonstrating the ability of EVK063 to attenuate cytokine production, *ex vivo* investigations will follow. Serial blood samples can be readily obtained after systemic administration of the compound. These samples will be stimulated with LPS and evaluated as previously described *in vitro*. *Ex vivo* studies will also allow evaluation of pharmacokinetic parameters and the efficacy of EVK063 following first pass effect. The *ex vivo* investigations will also provide the proper dose and interval for *in vivo* assessment. *In vivo* studies will provide the ultimate

test in the ability of EVK063 to not only inhibit cytokine production, but more importantly the manifestation of clinical signs.

References

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