

Current Understanding of Polymyxin B Applications in Bacteraemia/Sepsis Therapy Prevention: Clinical, Pharmaceutical, Structural and Mechanistic Aspects

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Abstract: Polymyxin B (PMB) belongs to a class of antibiotics discovered more than six decades ago. PMB was used for various bacterial infection threatening, in particular to sepsis. Its use, however, was abandoned because of the observation of severe side effects. In the last years this view changed due to the appearance of multi-drug resistant Gram-negative pathogens, which were resistant to most available antibiotics, leading to a re-evaluation of the polymyxin antibiotics (PMB and PME).

Although there is a large market potential for the development of drugs to fight sepsis, the available successful clinical strategies are very limited. The cause for this lies in the clinical failures of a number of drug candidates, which were tested in the last years. This was attributed to some extent to our elementary understanding of the pathophysiology of sepsis, to not optimally designed clinical trials and a lack of appropriate pre-clinical models to establish the proof of concept (POC). At that time there were just humble knowledge about the structural mechanisms involved in the advantageous aspects of PMB-endotoxin interactions to increase the knowledges outcome in sepsis therapy.

Therefore, the current paper describes the clinical aspects of PMB application in bacteraemia and sepsis therapy. However, the focus of the presented paper lies in the structural and mechanistic aspects of PMB-endotoxin (LPS: lipopolysaccharide) recognition and how this knowledge can be applied for the development or improvement of new clinical drug candidates to support sepsis therapies. Due to chemical similarities between PME and PMB, certain aspects of the use of PME as an antimicrobial agent and in sepsis therapy are considered and compared to PMB.

Keywords: Polymyxin, PMB, PME, endotoxin, lipopolysaccharide, LPS, sepsis, anti-infective agent, biophysics

INTRODUCTION

Polymyxins are a class of antibiotics (Fig. 1) discovered more than 60 years ago. It contains five different compounds (polymyxin A-E) from which only two have clinical relevance, namely polymyxin B (PMB) and polymyxin E (PME, also known as colistin) [1]. Polymyxin B was derived from *Bacillus polymyxa* in 1947. Two years later, using *Bacillus polymyxa* susp. *colistinus*, PME was made available [2].

The antimicrobial activity of polymyxins is focused against non-fermentative Gram-negative bacteria, which is summarized in Table 1. The PMB mechanism of action will be described below. PMB is used since more than five decades in topical and ophthalmic antibiotic preparations [4], but parenteral applications were not further considered because of early concerns about the toxicological potentials of PMB. The main side effects were nephrotoxicity and neurotoxicity [5]. However, in the last years the polymyxins were re-evaluated as antimicrobial agents. The main driving forces for this are a dramatic increase of problems encountered in the appearance of multi-drug resistance among clinically important Gram-negative bacteria and the absence of new

antibiotic drugs acting against these bacteria. The polymyxins are now more and more used via the parenteral route to treat infections caused by Gram-negative strains like *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, or *Klebsiella pneumoniae*, especially because they show the requested therapeutic effect and because the resistance of these bacteria against PMB and colistin is currently low [4, 6, 7].

However, the last issue is probably due to the limited application of polymyxins in the last years.

Zavascki and coworkers (2007) [8] have recently presented a critical review on the PMB treatment of multi-drug resistant pathogens and have summarised the main resistance mechanisms [9] to polymyxins in Gram-negative bacteria (Table 2)

PMB as sulphate salt has a bactericidal action against almost all Gram-negative bacilli (see Table 1) except the *Proteus* group. All Gram-positive bacteria, fungi, and the Gram-negative cocci *N. gonorrhoeae* and *N. meningitidis* are resistant [18].

Because polymyxins are seen as a “last option therapy” it is very important to apply polymyxins with great care to avoid any kind of generated bacterial resistance against these drugs. As a parenteral drug product, PMB is applied as water soluble PMB sulphate salt, whereas colistin is applied as a sodium salt of the prodrug colistin methanesulfonate (CMS).

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Table 2. Summary of Major Resistance Mechanisms to Polymyxins in Gram-Negative Bacteria

Bacterium	Resistance mechanism(s) to Polymyxin	References
<i>Pseudomonas aeruginosa</i>	Lipid A modifications with L-Ara4N controlled by PmrA/PmrB	[10]
<i>Salmonella enterica</i> serovar Typhimutium	Lipid A modification with both L-Ara4N and PEtn controlled by PmrA/PmrB	[11]
	The gene mig-14 is required for resistance but resistance does not implicate LPS modification	[12]
<i>Escherichia coli</i>	Lipid A modification with both L-Ara4N and PEtn controlled by PmrA/PmrB	[11]
<i>Klebsiella pneumoniae</i>	Increased production of capsule polysaccharide	[13]
<i>Burkholderia cenocepacia</i>	Complete requirement of LPS inner core oligosaccharide	[14]
<i>Helicobacter pylori</i>	Lipid A modification	[15]
<i>Yersinia pestis</i>	Lipid A modification with L-Ara4N controlled by PmrA/PmrB	[16]
<i>Vibrio cholerae</i>	Presence of outer membrane protein OmpU regulated by ToxR	[17]

L-Ara4N: 4-amino-4-deoxy-L-arabinose; PEtn: phosphoethanolamine

Both polymyxins, B and E, are evaluated and used for the treatment of patients (human and animals) with sepsis or septic shock. PMB is part of an extracorporeal device, where the peptide is chemically immobilized to fibre columns (see below).

Bacterial infections, e.g. induced by Gram-negative bacteria can be overcome by using antibiotics, which kill the bacteria. As a consequence of the permeation activity of the antibiotics, the bacterial cell is disrupted and the cell membrane components are released [19]. The cell membrane of bacteria is composed of a variety of diverse molecules such as glycolipids, among which are some extremely toxic for humans. To this class belong lipopolysaccharides (LPS) (Fig. 2), which play a decisive role for the induction of sepsis [20]. The more bacteria are killed, the more LPS is released increasing the severity of sepsis. [21]. Therefore, drugs like PMB act directly on LPS by binding and sequestering the endotoxin molecules [22-25] (see below). This property is not observed at that extent for other antibiotics [26, 27].

Although polymyxin B and polymyxin E (colistin) have been available for clinic applications for more than 50 years, neither polymyxin B nor polymyxin E have been subjected to drug development procedures required for the development of a state of the art pharmaceuticals [28, 29]. It is not sure whether this will happen, because the polymyxins are long off-patent, and the commercial incentive for industrial companies to sponsor preclinical and clinical research is quite limited.

As a consequence of this, the actual knowledge of pharmacokinetic, pharmacodynamic and toxicological properties of polymyxins are extremely scarce [30, 31].

Recently, Zavascki *et al.* (2008) have presented new data concerning the pharmacokinetics of PMB in critically ill patients. The drug was applied via the intravenous route (for more details see Zavascki *et al.* 2008) [8].

Furthermore, in order to improve the therapeutic efficacy of the drugs, less is known concerning the mechanism(s) of

action as well as the biophysical binding properties of these peptides with the endotoxin compounds. The last aspects are prerequisites for the development of "second" generation polymyxins bearing fewer side effects.

PATHOPHYSIOLOGY OF SEPSIS

Although the term sepsis is linked to modern intensive care medicine, it is long known. The word sepsis was introduced by Hippocrates (ca. 460-370 before Chr.) and its origin came from the greek σηπω (meaning "becoming rotten"). Ibn Sina (ca. 980-1037), also known as Avicenna, a famous Iranian philosopher and physician, observed, that the rottenness and decomposition of blood (septicaemia) is accompanied by high fever. The term sepsis as introduced in the ancient world was still used in this sense until the 19th century. A clear definition of sepsis was not available before the 90ties. In 1992, consensus definitions of sepsis and related terms were made available [32] and confirmed during the International Sepsis Consensus Conference held in 2001. Table 3 summarises the most important features for the definition of bacteraemia, sepsis and associated syndromes, which were mainly confirmed in 2005 [33].

Thus, an infection describes a microbial event, caused by e.g. bacteria, fungi or viruses, while sepsis is the host response to that infection, which is characterised by the release of many mediators (e. g. various interleukins and tumour necrosis factor- α). What makes the detection of sepsis quite challenging is the lack of specific clinical and laboratory features for sepsis. Considerable efforts are undertaken to find a sensitive and specific diagnostic marker for sepsis. Although some markers like procalcitonin and C-reactive protein (CRP) had this potential, recent studies showed that up to now none of the markers currently available is specific to sepsis [34]. These authors have reviewed the actual knowledge and techniques that can be used to facilitate the diagnosis of sepsis. The common clinical and laboratory features used for the detection of sepsis are summarised in Table 3.

Table 3. Consensus Definitions of Sepsis and Associates Syndromes (Adapted from the Consensus Conference Committee 1992 [32])

Term	Definition
Infection	Microbial phenomenon characterised by an inflammatory response to the presence of microorganisms or the invasion of normally sterile host tissue by those organisms
Bacteraemia	The presence of viable bacteria in the blood
SIRS (systemic inflammatory response syndrome)	The systemic inflammatory response to a variety of severe clinical symptoms, manifested by two or more of the following conditions: Temperature > 38 °C or < 36 °C Heart rate > 90 beats/min Respiratory rate > 20 breaths/min or $P_a(\text{CO}_2)$ < 32 Torr (< 4.3 kPa) White blood cell count > 12000 cells/mm ³ , < 4000 cells/mm ³ , or > 10 % immature (band) forms
Sepsis	SIRS that is caused by infection
Severe sepsis	Sepsis associated with organ dysfunction, hypoperfusion, or hypotension. Hypoperfusion and perfusion abnormalities may include, but are not limited to lactic acidosis, oliguria, or an acute alteration in mental status
Septic shock	Sepsis with hypotension, despite adequate fluid resuscitation, along with the presence of perfusion abnormalities that may include, but are not limited to, lactic acidosis, oliguria, or an acute alteration in mental status. Patients who are on inotropic or vasopressor agents may not be hypotensive at the time that perfusion abnormalities are measured
Hypotension	A systolic blood pressure of < 90 Torr (12.1 kPa) or a reduction of > 40 Torr (5.4 kPa) from baseline in the absence of other causes for hypotension
Multiple organ dysfunction syndrome	Altered organ function in an acutely ill patient, such that homeostasis cannot be maintained without intervention

Endotoxin (LPS) is a key initiator of sepsis. LPS is a component of the Gram-negative bacterial cell wall. Humans are especially sensitive to endotoxins and just tiny amounts (nano-molar range) of it are sufficient to induce an acute fever response [20]. The presence of higher endotoxin doses induces clinical syndromes known as septic shock (Table 3). The mortality rate in humans with endotoxic shock is about 50-60 % [35, 36].

Once the invading bacteria are lysed by antibiotics, the endotoxin is released into the systemic circulation, where it excites various cells like macrophages, monocytes or endothelial cells. In the blood stream, LPS binds to a protein termed LPS-binding protein (LPB), and the LPS-LPB complex interacts with CD14 on the surface of monocytes and macrophages. As a consequence a strong cellular activation is observed [37]. In addition to this, it is known that the LPS-

LPB complex can also interact with soluble CD14, which in this alliance (LPS-LPB-CD14 complex) can bind to another receptor on the surface of endothelial cells, which itself lacks the CD14 surface receptor. Evidence is also given that LPS is able to form complexes with serum lipoproteins like low density and high density lipoproteins (LDL and HDL), hemoglobin, and apolipoprotein A [38-40]. According to Lynn (1998) [29] these interactions provide an anti-endotoxin therapeutic option for the treatment of sepsis by eliminating LPS from the circulation.

As next step after the LPS-LPB complex has interacted with CD14 on the macrophage surface, a member of the Toll-like receptor (TLR) family, TLR4, is recruited [41]. Extensive studies on this receptor TLR4 have revealed that TLR4 is an essential signalling component of the LPS receptor indicating its role being highly specific [37]. TLR4 is a transmembrane protein with an extra-cellular domain containing multiple leucine-rich repeats (LRR). Another receptor of relevance is the intracellular Toll/interleukine receptor homology domain (TIR), recruited with the help of a secreted glycoprotein myeloid differentiation protein-2 (MD-2), which enhances LPS responsiveness [42]. As a consequence of the TLR4 recruitment, TLR4 induces the cellular machinery to produce various inflammatory cytokines like TNF- α (tumour necrosis factor), IL-1 (interleukin-1), IL-2 and IL-8.

Thus, endotoxins stimulate through complex pathways, the release of a variety of inflammatory mediators that serve to activate the host cellular defences. The most relevant inflammatory mediators are: TNF- α , IL-1, IL-2, IL-6, IL-8, IL-15, neutrophil elastase, IFN- γ (interferon), thromboxane, platelet activating factor (PAF), vasoactive neuropeptides, phospholipase A2, plasminogen activator inhibitor-1, prostaglandins, prostacyclin [36].

In addition to the production of these mediators, the macrophages produce reactive species like oxygen species or nitric oxide. The presence of high concentrations of reactive oxygen species, especially oxygen free radicals, provoke high fever and induce a reduction of blood pressure and un-hindered blood clotting throughout the body. As a result of hypotension and disseminated intravascular clotting, a decrease of the perfusion of vital organs is observed, followed by a strong ischemia of vital organs causing the failure and death of the organs. These clinical symptoms are indicative for the occurrence of endotoxic shock (for more details consult [20, 36, 37]).

ENDOTOXIN AND LIPOPOLYSACCHARIDE

Lipopolysaccharides (LPS) (Fig. 2) are a major constituent of the outer membrane of Gram-negative bacteria. Their unique structure contributes to the structural integrity of the outer cell membrane, protecting the bacterium from a number of chemical attacks. Due to historical reasons, the term LPS is often used exchangeable with endotoxin [20]. Because LPS molecules are negatively charged lipids, the negative cell membrane potential is increased, inducing a stabilisation of the overall membrane structure.

As can be seen from Fig. (2), LPS molecules belong to the class of glycolipids. They are composed of three distinct

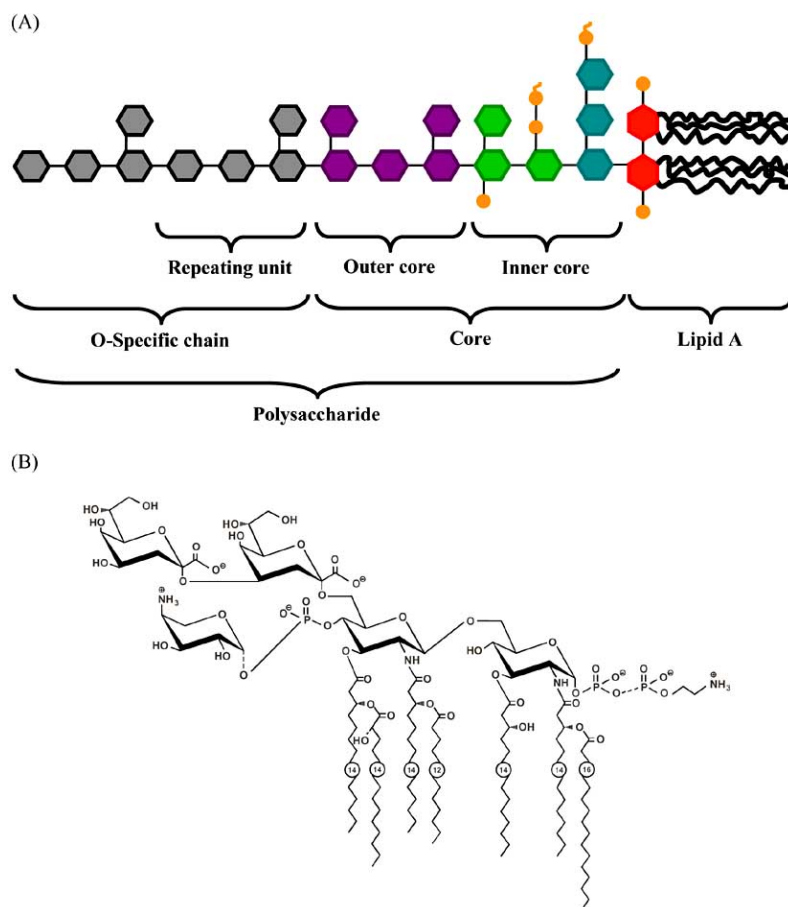


Fig. (2). (A) Chemical structure of Lipopolysaccharide (LPS), and (B) LPS: *Salmonella minnesota*. The colour represented in (A) denotes different sugar moieties (see text).

components of contrasting biophysical and physiological properties: 1) the variable, species-specific O-polysaccharide side chain, 2) the genera-specific core polysaccharide and 3) a structurally diverse structure known under lipid A [43].

The outer polysaccharide side chain (O) is also referred as the O-antigen of the bacteria. The composition of the O side chain varies between different Gram-negative bacterial strains, and determines the serotype specificity of each bacterial strain. The O-polysaccharide side chain varies in the composition and number of sugars within the unit, varies in the chemical link of the sugars as well as the number of repeating units within the O-polysaccharide region. The presence or absence of O chains determine whether the LPS is considered rough or smooth. The discrimination between smooth and rough LPS is determined by the structure of the O-polysaccharide side chain; full length O-chains renders the LPS smooth while the absence or reduction of O-chains make the LPS rough. As a consequence of the difference in the outer sugar side chain, the impact of antibiotics is different. Bacteria with a cell membrane composed of rough LPS are more sensitive to the penetration of hydrophobic antibiotics, because rough LPS is more hydrophobic due to the reduced O-polysaccharide side chain.

The core polysaccharide is composed of unusual sugars (e.g. 3-deoxy-D-manno-oct-2-ulopyranosonic acid, Kdo, 2-keto-3-deoxyoctulosonate and heptose) (Fig. 2A). The num-

ber of sugars of the core polysaccharide lies between 10-12 sugar units [44]. Compared to the O-polysaccharides which shows a high structural variability, the sugar composition of the core is less variable within a genus. The heptose residues are often substituted by phosphate, pyrophosphate and diphosphoethanolamine. For nearly all LPS, the core oligosaccharide is attached to lipid A through an acidic sugar (Kdo). Lipid A is mainly responsible for the toxicity of Gram-negative bacteria, called its 'endotoxic principle' [43].

The lipid A structure functions as an anchor and is embedded into the outer membrane while the rest of the LPS is directed into the environment. Lipid A from enterobacterial strains is composed of a bisphosphorylated diglucosamine disaccharide with six fatty acid residues ester- and amide-linked to the sugar backbone. Variabilities of lipid A were observed in the pattern of substitution of the two lipid A phosphates, the type of fatty acid chains and the degree of acylation. This is the key of its toxicity. The so-called LPS 'endotoxic principle' established by the Borstel scientists [43-51], has been shown to be consistent with *Escherichia coli*-type LPS, whereas deviation from this, for example by strains synthesizing pentaacylated lipid A, may lead to agonistic inactivity. Interestingly, this compound is even able to block the activity of LPS, i.e., it acts antagonistically [48, 49].

This lipid A (Fig. 2A) adopts a cubic inverted aggregate structure from which a conical shape of the molecule can be deduced, whereas the tetraacyl lipid A precursor IVa adopts a cylindrical shape and is endotoxically inactive, but antagonizes active LPS [52].

These super-structural assemblies play a key role for the induction of the cellular responses [43, 52].

CLINICAL MANAGEMENT OF SEPSIS

A number of strategies were investigated to treat sepsis patients (Table 4) [53]. Just to refer to some few strategies considered: development of anti-endotoxin antibodies for the neutralisation of endotoxins, blockage of tumour necrosis factor at the tissue level, development of an interleukin-1 receptor, cyclooxygenase or thromboxane antagonists, or the development of endotoxin-neutralising peptides (Table 4). However, none of these therapies showed an effective success in sepsis treatment [54, 55].

The identification of sepsis is extremely complex in critical care units, because the signs and symptoms are highly variable [56]. The observed symptoms are non-specific but together contribute to the picture of systemic illness. Modern clinical management of sepsis is focussed on four levels. First, the septic focus, if identified, must be removed. This is an important intervention, because it allows a strong reduction of the invading bacteria. Furthermore, abscess drainage has to be performed as well as the removal of necrotic tissue. The second step involves the treatment of the infection by antimicrobial agents. As described above, this intervention can increase the sepsis syndromes due to the fact that the antimicrobial agent lyses the bacteria and with this endotoxins are released. The third sepsis management step consists of supportive measures, which includes hemodynamic resuscitation, transfusion, organ support, catecholamine therapy as well as artificial nourishment. The last aspect of sepsis management considers the modulation of the host response (for more details consult Weigand *et al.* 2003 [57], Dellinger *et al.* 2008 [58]). Fig. (3) represents some strategic approaches for the prevention and treatment of sepsis (adapted from [59]). Recently, Landman *et al.* (2008) have reviewed the pharmacokinetic as well as the dosage guidelines for using PMB in clinical trials. For more details, please consult this excellent report [60].

CHEMICAL PROPERTIES OF POLYMYXINS

Polymyxin B is a cyclic lipo-decapeptide antibiotic, with seven amino acids in the peptide ring, containing a tripeptide side chain and a fatty acid tail (Fig. 1). The difference between PMB and colistin is quite low; they differ just by one amino acid in the cycle peptide [28]. Both polymyxins are multicomponent antibiotics. This is due to the fact that there are differences between the hydrocarbon chains (6-methyl-octanoic acid or 6-methylheptanoic acid).

Under physiological pH conditions, the five primary amine groups on the amino acids are ionized. It results a positively charged (theoretically five fold positive charged) polypeptide. The charges as well as the location of the charges are crucial for the binding of PMB to endotoxins (see below).

Table 4 Treatment Strategies Investigated for Sepsis (Adapted from Wheeler and Bernard 1999 [54])

Strategy	Rational for the Therapeutic Application
Antiendotoxin antibodies	Endotoxin neutralisation
Tumour necrosis factor antibodies	Blockage of tumour necrosis factor at the tissue level
Soluble tumour necrosis factor receptor entity	Blocking of tumour necrosis factor at the tissue level
Interleukin-1 receptor antagonists	Reduction and inhibition of interleukin-1 on cellular receptor
Interleukin-1 antibodies	Prevention of the interaction with interleukin-1 receptor
Antioxidant compounds	Neutralisation effects of oxidant-mediated tissue injury
Anticoagulants	Inhibition of microtubuli formation and injury due to tissue ischemia and reperfusion
Bactericidal permeability-increasing protein	Bacteria kill and endotoxin neutralisation
Peptides (e.g. polymyxin B)	Bacteria lysis and endotoxin neutralisation
Bradykinin-receptor antagonists	Prevention of vasoactive effects of bradykinin
Cyclooxygenase antagonists	Blockage of pyrogens, endotoxins, thromboxane, and prostacyclin
Thromboxane antagonists	Inhibition of inappropriate vasoconstriction and platelet aggregation
Platelet activating factor antagonists	Blockage of platelet activation and inflammatory lipid release
Inhibitors of leukocyte-adhesion molecules	Prevention of endothelium-leukocyte interaction
Nitric oxide antagonists	Restoration of appropriate vasoregulation

PMB, administered parenterally as a sulphate salt, is available as a mixture of at least four components with PMB1 and PMB2 being the most prominent (see Fig. 1). The four components differ from each other only by the acyl chain composition with PMB1: 6-methyloctanoic acid, PMB2: 6-methylheptanoic acid, PMB3: octanoic acid and PMB4: heptanoic acid. PME is predominately administered parenterally as the sodium salt of colistinmethanate sodium (CMS). The reason of using this PME derivative was the assumption that this might reduce the side effects of PME as sodium salt. Since recently, it is known that when CMS is applied via the parenteral route, the five methanesulphate groups of CMS undergo hydrolytic cleavage *in vivo*. As a consequence pure PME (colistin) is generated, which is the

active ingredient. Bergen *et al.* (2006) [61] have demonstrated that the antibacterial activity of CMS against *Pseudomonas aeruginosa* is nearly not existing. Therefore, CMS acts as an inactive prodrug of PME (colistin).

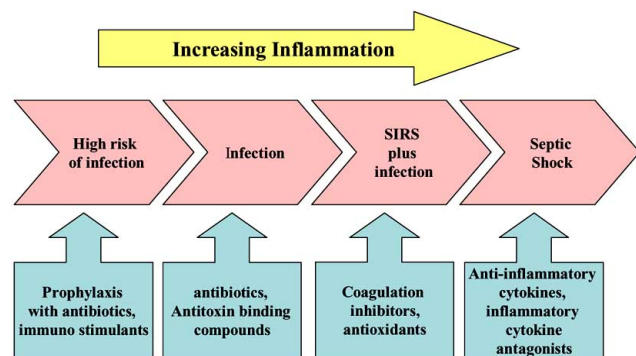


Fig. (3). Strategies for the prevention and treatment of sepsis (adapted from [59]).

(SIRS: Systemic Inflammatory Response Syndrome)

TOXICITY

In the early years the main reason for the abandonment of polymyxin as a parenteral drug was due to severe toxicological side effects [6]. In the older literature the following toxicities were reported: nephrotoxicity, neurotoxicity and neuromuscular blockade [62]. However, in the studies performed in the last few years the therapeutic potential of polymyxin was reconsidered and as a result of careful dosing and application scenarios in the clinical treatment, the toxicological assessment of PMB as described in the older studies could not be confirmed. The reason for this is probably because the observed toxicity with the earlier clinical applications was at least in part results of a limited understanding of the pharmacokinetics, pharmacodynamics and toxicodynamics of PMB. Recent studies have shown that parenteral applications of polymyxins are better tolerated as expected [31].

Although polymyxins have the potential being nephro- and neurotoxic, these side effects are usually reversible upon ceasing polymyxin administration. Both side effects are dose-dependent, thus a reduction or avoidance of nephro- and neurotoxic can be achieved by applying a suitable dosing regime. Furthermore, a thorough body fluid and electrolyte management during the treatment is required. Although cases of neuromuscular blockade and apnoea were described in the older literature, these side effects were not reported from recent studies [5].

The polymyxin antimicrobials are poorly absorbed from the gastrointestinal tract. Colistin, as an example, concentrates in the liver, kidney, muscle, heart, and lungs but does not consistently cross the blood–brain barrier in non-inflamed meninges. Polymyxins are excreted primarily by the kidneys. Pharmacokinetic studies in the 1960s demonstrated that the serum half-life of these drugs increases from 6 hours or less in individuals with normal renal function to 48 hours or more in anuric patients

In order to avoid toxic side effects, accurate dosing is of importance and also the corresponding formulation. The dosage of intravenous polymyxin B as recommended by the

manufacturer [18] is 1.5 to 2.5 mg/kg * d (15,000–25,000 IU/kg/d) divided into two equal doses for adults and children older than 2 years with normal renal function [8].

One milligram of polymyxin B equals to 10,000 IU. Dosage adjustments for polymyxin, especially colistin, are recommended for patients with mild to moderate renal dysfunction. Due to similarities between both polymyxins, this should also be considered for PMB. However, recommendations for dosage adjustment of polymyxin B in the presence of renal impairment have actually not been well established. Additional information on dosing of polymyxins have been reported recently [2, 28, 31, 62].

COMMERCIAL AVAILABILITY OF PMB FOR CLINICAL USE

One commercial source for clinical application is provided by Bedford Laboratories. PMB for injection, as sulphate salt, is derived from *B polymyxa* (*B aerosporous*). PMB sulphate is the sulphate salt of Polymyxins B₁ and B₂ (Fig. 1), which are produced by the growth of *Bacillus polymyxa* (Prazmowski) Migula (Fam. Bacillacea). Based on the information provided by Bedford Laboratories (<http://daily.med.nlm.nih.gov>, where an actual version with handling information, revised on 01/2008, is available from Bedford Laboratories), PMB sulphate has a potency of not less than 6000 PMB units per mg, calculated on the anhydrous basis.

Each vial contains 500,000 polymyxin B units for parenteral or ophthalmic administration. PMB for injection is formulated as a powder, suitable for preparation of sterile solutions for intramuscular, intravenous drip, intrathecal, or ophthalmic use. The storage recommendations, before reconstitution, are controlled room temperature between 15° to 30 °C and protection from light is advised.

With regards to the stability of the final drug product, Bedford Laboratories reports that aqueous solutions of polymyxin B sulphate, stored under refrigeration, (2 - 8 °C) may be stored up to 12 months without significant loss of potency. In the interest of safety, solutions for parenteral use should be stored under refrigeration and any unused portion should be discarded after 72 hours. Polymyxin B sulphate should not be stored in alkaline solutions since they are less stable (for more details, consult the homepage of <http://daily.med.nlm.nih.gov>, where an actual version with handling information, revised on 01/2008, is available from Bedford Laboratories [18]).

For intravenous solutions, Bedford Laboratories recommend to dissolve the drug with 5% dextrose injection solution, for intramuscular use sterile water for injection or sodium chloride injection or 1% procaine hydrochloride injection and for ophthalmic application sterile water for injection or sodium chloride injection USP can be used.

Dosing of the polymyxins in the clinical trials is somewhat confusing [4] and not always clear. For PMB, dosages have frequently been given in terms of equivalent weights of pure polymyxin B base. According to Bedford Laboratories, each milligram of pure polymyxin B base is equivalent to 10,000 units of polymyxin B.

PME is mostly used as sodium colistinmethanate (for more details see Horton *et al.* 1982 [63]).

ANTIMICROBIAL ACTIVITY

The reason for the use of the polymyxin antibiotics were due to their broad spectrum of activity. Table 1 summarises the antimicrobial activity of PMB [3].

Hogardt *et al.* (2004) [64] showed that PMB exhibits good activity against *P. aeruginosa* at MIC₉₀ ≤ 4 mg/l which is in accordance to the data published by Gales *et al.* 2006 [3].

So far, most investigations on the pharmacodynamics of the polymyxins have focused on colistin, and less is known about the pharmacodynamics of PMB [65]. Better understanding of the pharmacodynamics of polymyxin B may help to determine its exact dose rationale to optimize patient outcomes and avoid resistance. Recently, Tam *et al.* (2005) [30] examined the pharmacodynamics of polymyxin B against *P. aeruginosa* and suggested that the bactericidal activity of this regimen was concentration-dependent and seemed to be related to the ratio of the area under the concentration-time curve to the MIC. Investigations for the determination of the optimal dosage of these regimens in different subpopulations of patients are, however, urgently required.

In the last years antimicrobial resistance was observed for both polymyxins, since Gram-negative bacteria have developed various resistance mechanisms to overcome the antimicrobial activity (for more details see Table 2 and references cited therein) [66]. Ko and coworkers (2007) [67] have investigated the antimicrobial resistance of PMB and PME in clinical isolates of *Acinetobacter spp.* They have collected 265 isolates of *Acinetobacter spp.*, which were identified to species level using partial *rpoB* gene sequences. About 81 % of the isolates were *Acinetobacter baumannii*. Out of these about 18 % and 28 % were resistant to PMB and PME, respectively. Antoniadou *et al.* (2007) [68] have also reported about PME resistant isolates of *Klebsiella pneumoniae* which were encountered in intensive care unit patients.

APPLICATION OF POLYMYXINS IN BACTEREMIA/SEPSIS

Michalopoulos and Falagas (2008) [4] have recently summarised the use of PME and PMB in critical care units, because these peptides were mainly administered for the treatment of life-threatening nosocomial acquired infections caused by multiple-drug resistant Gram-negative pathogens, like *Acinetobacter sp.*, *P. aeruginosa*, *Klebsiella sp.*, and *Enterobacter sp.* in adult patients, due to the lack of other therapeutically efficient agents [19, 69]. Recent reports have also demonstrated the use of these agents, especially PME as intravenous administration in children [28, 70, 71].

The clinical and microbiologic efficacy and safety profile of PMB in the treatment of multidrug-resistant Gram-negative bacterial infections of the respiratory tract was re-examined retrospectively by Sobieszczyk *et al.* (2004) [72]. They presented a combination therapy study with PMB for the treatment of multidrug-resistant Gram-negative respiratory tract infections. In their study, 25 critically ill patients were treated. The patients received a total of 29 courses of

PMB, which was administered in a combination study with another antimicrobial agent. They used two administration routes. The patients were treated with intravenous and aerosolized PMB, at a mean PMB duration therapy of 19 days. At the end of the treatment a mortality of 21 % was observed, and overall mortality at discharge was 48 %. Nephrotoxicity was observed in 3 patients (10 %). However, this side effect did not result in discontinuation of the therapy. The outcome of the study presented by Sobieszczyk and colleagues (2004) [72] was that PMB combined with other antimicrobials can be considered as a reasonable and safe treatment option for multidrug-resistant Gram-negative respiratory tract infections in the setting of limited therapeutic options.

The study published by Holloway *et al.* (2006) [73] investigated the administration of intravenous PMB in 29 critically ill patients with infections caused by multidrug-resistant *A. baumannii*. The observed clinical cure was 76 %, whereas crude mortality rate was 27 %. Sarria and colleagues (2004) [70] reported their experience from the use of intravenous PMB in a patient with *A. baumannii* sepsis and acute renal failure that required continuous hemodialysis and were able to present a successful treatment.

Recently, an intravenous PMB application was presented for the treatment of 74 patients infected by hospital-acquired multidrug-resistant *Pseudomonas aeruginosa* [74]. A favourable outcome for 48 % of the treated patients was observed, which led to the conclusion that PMB is a reliable antimicrobial drug, but only as salvage therapy, for nosocomial pneumonia caused by multidrug-resistant *Pseudomonas aeruginosa*.

The data presented by Pastewski *et al.* (2008) [75] suggest that PMB may be effective for multidrug-resistant Gram-negative bacteraemia and urinary tract infections for patients with limited therapeutic options, but they pointed out that precautions should be taken by the correct dosing in order to avoid toxicity, especially nephrotoxicity.

Examples are also known where a therapeutic difference was observed between PME and PMB. Systemic colistin (PME) has shown efficacy against multidrug-resistant *Pseudomonas aeruginosa* and *Acinetobacter spp.*, but it has presented poor results in pneumonia. Aerosolized polymyxin (PMB) in cystic fibrosis patients led to good results. Pereira *et al.* (2007) [76] used inhaled PMB to treat respiratory infections by multidrug-resistant Gram-negative bacilli. Nineteen patients were treated with inhaled polymyxin B: 14 had pneumonia, most of which had previously failed treatment with intravenous PMB, and 5 tracheobronchitis. Inhaled PMB was given at a dose of 500,000 IU twice a day after an aerosolized β_2 -agonist. In pneumonia, inhaled and intravenous PMB was administered together. 89 % of the patients were in the intensive care unit. Sixteen infections (84 %) were caused by *P. aeruginosa*, while *Klebsiella pneumoniae*, *Alcaligenes xylosoxidans*, and *Burkholderia sp.* caused one infection each. In the 14 pneumonia cases, the median of previous use of intravenous polymyxin B was 20 days (range 0-32), whereas inhaled polymyxin B was used for a mean of 14 days (range 4-25). Cure occurred in 10 (53 %) patients, improvement in 8 (42 %), and failure in 1. Nine patients died during hospitalization (all with pneumonia). Adverse events

occurred in 4 patients without interruption of inhalation. The study published by Pereira *et al.* (2007) [76] is the largest report using inhaled PMB to treat nosocomial pneumonia by multidrug-resistant Gram-negative bacilli that had failed intravenous polymyxin B. It was also effective alone in *P. aeruginosa* tracheobronchitis. This report highlights that the route of application also determines the outcome of the therapy.

Studies are known, where both polymyxins are applied for the treatment of bacteraemia and sepsis [31]. Zhou *et al.* (2007) [77] have demonstrated that polymyxin was effective in the management of multidrug-resistant *Pseudomonas aeruginosa* pulmonary infections in patient with the highly pathogenic avian influenza (H5N1) infection. In this study both polymyxins were used. PMB was subsequently administered intramuscularly or intravenously combined with PME aerosol therapy.

The use of colistin in bacteraemia and sepsis therapy can be summarised as follows. Colistin applied intravenously in intensive care units patients with sepsis showed in 73 % of the treated patients a clinical response [78]. They reported a deterioration of renal function in ca. 14 % of patients, whereas survival at 30 days was 57.7%. Karabinis *et al.* (2004) [79] described the successful management of a patient with bacteraemia/septic shock caused by *K. pneumoniae*. Again PME was administered via the intravenous route at a dosage of 9 million IU/d (2.5 mg/kg, divided into three doses). Michalopoulos and colleagues (2005) [69] used a continuous intravenous application of PME in a critically ill patient with bacteraemia caused by a multiresistant *A. baumannii* strain susceptible only to PME. With the used approach, they were able to cure the patient from this life-threatening infection. The same group [80] also reported that the mortality rate was acceptable (35.7%) in critically ill patients with ICU-acquired bacteraemia caused by MDR *A. baumannii* who received intravenously colistin and meropenem, based on sensitivity tests and MICs. On the contrary, the mortality rate was 56% in the group of patients with bacteraemia caused by *A. baumannii* strains susceptible only to PME (see also Michalopoulos and Falagas 2008 [4]).

Michalopoulos and Falagas (2008) [4] have recently mentioned for polymyxin E that „that the selective pressure caused by extensive or inadequate colistin use may have contributed to the emergence of colistin resistance among *P. aeruginosa*, *A. baumannii*, and *K. pneumoniae* isolates”, potentially increasing morbidity and mortality rates in critically ill patients and necessitating prudent use of colistin [68]. For this reason, the empiric use of colistin should be limited to institutions in which there is recognized infection caused by multidrug-resistant Gram-negative bacilli [79].

This is also true for PMB. It is very crucial to use these antimicrobial agents with care and only where it is really appropriate in order to avoid multi-drug resistance [81].

APPLICATION OF POLYMYXINS IN AN EXTRACORPOREAL MEDICAL DEVICE

An extracorporeal hemoperfusion device (TORAY-MYXIN) based on PMB was first developed in 1994. It is designed for the selective blood purification from endotoxins

via direct hemoperfusion. Toraymyxin is made up of polystyrene-derivative fibres to which the peptide is covalently bound on the surface of an insoluble carrier material inside the cartridge [82-84]. According to Teramoto *et al.* (2002) [85] Toraymyxin is composed of an islands-in-a-sea-type composite fibre with the island ingredient comprising polypropylene, and the sea ingredient comprises a polystyrene derivative. It is prepared from the corresponding polystyrene fibre, which is amidomethylated with *N*-methylol- α -chloroacetamide, and the resulting fibre is incubated subsequently in the PMB aqueous strongly alkaline solution (pH 9 to 13). As a consequence, PMB is chemically bonded to α -carbon of the acetyl residue in poly(4-chloroacetamidomethyl styrene) of PMX-F by replacing chlorine. PMB is covalently bounded at a weight ratio of 0.5 w% [86].

The advantage of this extracorporeal device is due to the fact that the endotoxin can be inactivated in the blood without exerting its toxicity on the brain and kidney.

Direct hemoperfusion using such polymyxin B-immobilized fiber column (PMX; Toray Industries Inc., Tokyo, Japan) has been tested and used since about 15 years for the treatment of septic shock.

Ruberto *et al.* (2007) [87] have recently analysed the efficacy, safety and clinical effects of direct hemoperfusion with an immobilized polymyxin-B fibre column (DHP-PMX) in solid organ transplanted patients with severe sepsis or septic shock. 15 patients (mean age 55 years old) were considered which developed severe sepsis or septic shock after kidney or liver transplantation. For all patients, Gram-negative bacteria were detected. They were treated using the conventional approach, namely: antibiotic therapy, vasopressor or inotropic agents, and ventilation support. Additionally, each patient was treated three times using DHP-PMX treatment. Ruberto and coworkers (2007) [87] observed no adverse events for the 15 treated patients and they concluded that the use of DHP-PMX in association with conventional therapy may be an important aid in patients with sepsis.

A beneficial outcome was also described for patients with severe sepsis or septic shock which underwent a liver transplantation by applying a direct DHP-PMX hemoperfusion [88].

DHP-PMX as a treatment option is used in a number of clinical cases. Kakugawa *et al.* (2008) [89] recently reported on the successful DHP-PMX treatment outcome of a patient undergoing a rapidly progressive interstitial pneumonia associated with clinically amyopathic dermatomyositis.

Murakami *et al.* (2007) [90] observed that it is important to initiate early in suspected septic shock patients the simultaneous DHP-PMX and drug treatment in order to facilitate hemodynamic improvement, which is beneficial for the patient.

A number of molecular factors affect the clinical outcome during sepsis or septic shock. Using the DHP-PMX approach after a continuous hemodiafiltration with polymethylmethacrylate membrane hemofilters for the treatment of septic shock patients, also a beneficial improvement of critical laboratory parameters like systolic blood pressure and an improved Pao₂/Fio₂ ratio after treatment has been shown [91, 92].

The activity of proapoptotic circulating factors play a crucial role for patient which were injured by a Gram-negative sepsis-induced acute renal failure. Cantaluppi *et al.* (2008) [93] tested the hypothesis that the extracorporeal therapy with DHP-PMX may prevent Gram-negative sepsis-induced acute renal failure by reducing the activity of proapoptotic circulating factors. Using cultured renal cells, a significant decrease of plasma-induced proapoptotic activity was observed after DHP-PMX treatment, which confirmed their approach.

Another factor of relevance during sepsis, is the activation of neutrophils that injures tissues and organs. From literature it is known that blood purification therapies such as continuous veno-venous hemofiltration (CVVH) and direct hemoperfusion with polymyxin-immobilized fibre (DHP-PMX) have been used for the treatment of sepsis and septic shock, however, the effects of such therapies on neutrophil activation have been poorly understood. Naka *et al.* (2006) [94] have therefore focused on this aspect, and have evaluated neutrophil reactive oxygen species, in particular the H₂O₂ production, in the pathophysiology of sepsis or septic shock and the effect of continuous veno-venous hemofiltration or direct hemoperfusion with polymyxin-immobilized fibre on neutrophil reactive oxygen species. First of all, Naka and coworkers (2006) [94] found that patients with sepsis or septic shock had significantly higher levels of neutrophil reactive oxygen species compared with normal volunteers. Neutrophil reactive oxygen species did not change over time in patients treated either with continuous veno-venous hemofiltration or without this treatment. However, applying direct hemoperfusion with polymyxin-immobilized fibres to patients with septic shock, they were able to demonstrate a significant inhibition of neutrophil reactive oxygen species of ca. 30 %.

Another factor of relevance is 8-hydroxy-2'-deoxyguanosine (8-OHdG). In healthy volunteers the concentrations of 8-OHdG were 5.5 ng/mg creatinine, whereas in septic shock patients it was 38.0 ng/mg [95]. The study demonstrates that urinary 8-OHdG levels correlated significantly with plasma endotoxin levels, the Acute Physiology and Chronic Health Evaluation score and the Sepsis-related Organ Failure Assessment score (all with $p > 0.01$). Nakamura *et al.* (2006) [95] were able to provide evidence that urinary 8-OHdG levels, which are believed to be associated with septic shock, decreased effectively using polymyxin B-immobilized fibre (PMX-F) haemoperfusion.

Beyond the binding of endotoxins to PMB, also other molecules such as endocannabinoids bind strongly to PMB. From the last class arachidonylethanolamide (AEA) and 2-arachidonylglycerol (2-AG) are of interest because they are involved in septic shock. The prostaglandin 8-epi prostaglandin F₂ α (F₂-isoprostane) is used as a biomarker of oxidative stress in biological systems. Kase and co-workers (2008) [96] have therefore evaluated whether a DHP-PMX hemoperfusion therapy would decrease serum levels of endocannabinoids. Twenty-six patients with septic shock, including those with septic shock induced by peritonitis, underwent laparotomy for drainage. Two groups of patients were formed: patients whose mean arterial blood pressure had increased more than 2.6 kPa (=20 mm Hg) (responder

group; N = 13); and patients whose mean arterial blood pressure did not increase or had increased no more than 2.6 kPa (non-responder group; N = 13). They observed no changes of arachidonylethanolamide after DHP-PMX in either the non-responder or responder groups. However, the levels of 2-arachidonylglycerol decreased significantly after DHP-PMX hemoperfusion therapy in the responder group, but not in the non-responder group.

F₂-isoprostane gradually increased after DHP-PMX treatment; on the other hand, levels of F₂-isoprostane remained constant in the responder group. Patients with septic shock are under considerable oxidative stress, and 2-arachidonylglycerol plays an important role in the cardiovascular status of these patients. Kase *et al.* (2008) [96] concluded that the removal of 2-arachidonylglycerol by DHP-PMX benefits patients with septic shock by stabilizing cardiovascular status and decreasing long-term oxidative stress.

The efficacy of PMX has been proven in studies with a uniform case definition and without any other blood purification techniques. However, based on the knowledge that in some studies favourable effects of direct hemoperfusion with polymyxin-B-immobilized fibre columns (PMX) for the treatment of septic shock have been reported, Shimizu and collaborators (2008) [97] have recently demonstrated that a direct hemoperfusion with PMX improves septic hypotension and reduces inflammatory mediators in septic patients with colorectal perforation. In their investigation, 52 patients with severe sepsis or septic shock secondary to colorectal perforation were treated with DHP-PMX. A number of clinical parameters were considered (hemodynamic alterations and plasma concentrations of endotoxin, interleukin (IL)-1 β , IL-1 receptor antagonist (IL-1Ra), IL-6, IL-8, and IL-10) following PMX treatment. Shimizu *et al.* (2008) [97] observed a significant reduction in plasma endotoxin in the survivors immediately after DHP-PMX treatment compared to before treatment. Furthermore, inflammation factors like IL-1 β , IL-1Ra, and IL-8 were significantly reduced during a 2-h interval of PMX. They concluded from their results that DHP-PMX treatment appears to adsorb endotoxin and additionally a modulation of circulating cytokine is observed, both effects being beneficial for the treatment of the patients.

Kushi and colleagues (2008) [98] investigated the effects of DHP-PMX treatment with regards to the gastric intramucosal pH (pHi) for patients who underwent early goal-directed therapy within 6 h of a diagnosis of sepsis or septic shock. Their findings suggest that DHP-PMX improves gastric intramucosal pH with normalized values from 48 h onwards.

The studies presented above have clearly shown that direct hemoperfusion using a polymyxin B-immobilized fibre column has been used successfully for the treatment of septic shock. Septic shock is a condition associated with diffuse coagulopathy and multiple organ failure, and frequently ends in death. On the other hand, the effectiveness of continuous hemodiafiltration using a polymethylmethacrylate membrane hemofilter (PMMA-CHDF) for critically ill patients has also been reported [92]. 27 septic shock patients were treated using the DHP-PMX approach. The patients, except for the nine in whom continuous hemodiafiltration (CHDF) was not performed after DHP-PMX, were divided into two groups:

namely, a group in which PMMA-CHDF therapy was added after DHP-PMX (11 cases), and a group in which continuous hemodiafiltration using a polyacrylonitrile membrane hemofilter (PAN-CHDF) therapy was added after DHP-PMX (7 cases). The outcomes in the two groups were compared. The average Acute Physiology and Chronic Health Evaluation (APACHE) II score and the average sepsis-related organ failure assessment (SOFA) score were not significantly different between the two groups. The PMMA-CHDF group showed significantly better outcomes, with significant improvements of the serum PAI-1, protein C, IL-6 and N-arachidonoylethanolamine (AEA) levels. Based on their results Sakamoto *et al.* (2008) [92] concluded that PMMA-CHDF may be more effective than PAN-CHDF in the management of septic shock.

A number of studies are still devoted to investigate this extracorporeal medical device for the removal of endotoxins from the blood [99-102].

VETERINARY APPLICATIONS

PMB and DHP-PMX has been tested and evaluated in a number of animals models like rats [103, 104], dogs [105, 106], pigs [107], sheep [108] and horses [109], to inhibit LPS-induced fever, because PMB binds to the lipid A of bacterial endotoxin. These data are of relevance for the pre-clinical evaluation of the therapy.

However, there is a certain interest to develop a therapy against endotoxemia in horses [108-110], because there is a lack of a safe, affordable and effective treatment for endotoxemia in horses in order to reduce the incidence of this potentially fatal condition.

Results, presented by various studies [111, 112] suggest that PMB is a safe, effective inhibitor of endotoxin-induced inflammation in healthy horses.

MacKay *et al.* (1999) [113] determined the efficacy of polymyxin B-dextran 70 (PBD) formulation for the treatment of endotoxemic horses and found that when used in combination with a cyclooxygenase-inhibiting drug, PBD has potential for treatment of horses with endotoxemia.

PME has also been tested in various animals like dogs. Sentürk (2005) [114], as an example, found that PME has an anti-endotoxic effect and can be used safely for dogs with endotoxemia.

PHARMACEUTICAL CONSIDERATIONS

Systemic applications of PMB have been strongly limited, especially for the parenteral route, due to its high toxic potential. The entrapment of antibiotics in liposomes or other carrier systems is known to enhance their antimicrobial activities while minimizing their toxic effects.

This is also known for oral application, because Coppi *et al.* (2008) [115] showed that the use of alginate/chitosan microparticles to target the lymphatic system could improve safety when administering PMB orally (see also [116]).

Alipour and colleagues (2008) [117] have recently investigated the formulation and incorporation of PMB into liposomes. They have prepared different pseudobinary lipid

systems composed of a phosphatidylcholine and cholesterol. The entrapment efficiency of sonicated liposomes were dependent on the acyl chain composition of the phospholipids, being six-fold higher for saturated compared to unsaturated phospholipids acyl chain composition. It was also observed, that the entrapment efficiency was dependent on the liposomal preparation, because for extruded liposomes, the entrapment efficiency was higher for lipids containing unsaturated acyl chains.

The drug release was also different when saturated or unsaturated lipids were used, with a faster release kinetics for the saturated phospholipids formulation. Alipour *et al.* (2008) [117] focussed on the antimicrobial activities of the PMB liposome formulations, showing the minimal inhibitory concentrations of sonicated saturated liposomes against Gram-negative strains were generally lower when compared to free polymyxin B. This is explained by the fact that the penetration of PMB into a resistant strain of *Pseudomonas aeruginosa* was higher following its administration as a liposomal formulation as compared to its conventional form. The combination of free PMB and with liposomal containing PMB formulations had an antibacterial activity similar to that of free antibiotic. The author concluded that an "incorporation of polymyxin B in liposomes could be useful in the management of Gram-negative infections induced by these microorganisms."

Another study presented by McAllister *et al.* (1999) [118] were focused on the observation that the pulmonary residence time of PMB is substantially increased when administered as a liposomal formulation. The main criteria for the use of liposomal PMB formulations to improve the treatment of cystic fibrosis lung infections, is an unaffected antimicrobial activity of PMB for the liposomal formulation. Therefore, McAllister *et al.* investigated whether the microbial activity against *Pseudomonas aeruginosa*, was retained for liposomal preparation versus the free drug. They were also interested in the role of liposomal surface characteristics in determining interactions with bacterial cell surfaces. They showed that the encapsulation efficiency was dependent on the charge of the used excipients. Using positively charged amphiphiles, PMB encapsulation was reduced compared to neutral or negatively charged lipids. Concerning PMB antimicrobial activity, it was found that antimicrobial activity was retained after encapsulation. Using a PMB concentration of 0.3 mg/l, McAllister *et al.* (1999) [118] showed that positively as well as negatively charged liposomal PMB formulations, and the free drug, killed all cells after 1 h, whereas the use of neutral liposome formulations did not significantly decrease the surviving cell fraction. The reason for the differences in the minimal inhibitory concentrations of various liposomal formulation could be correlated to the free drug concentration, which was obtained through the release of entrapped PMB. Enhanced activity was only observed for formulation composed of positively charged excipients, which may be related to favourable electrostatic interactions between the cells and the liposomes. In summary, they conclude that "liposome encapsulation of PMB was not detrimental to antimicrobial activity, and liposome surface properties and release characteristics were important in determining interactions with bacterial cells". Other drug delivery

livery systems of interest for the formulation of PMB are lipid nanoparticles and nanoemulsions [119].

For ophthalmic PMB formulations the choice of the correct preservative is of high relevance to maintain PMB activity [120, 121].

Additional aspects of pharmaceutical relevance are related to analytics, stability and purity of the drug product (for more details see [122]).

STRUCTURAL AND MECHANISTIC INTERPRETATIONS

Dispersed in water, LPS molecules form down to extremely low concentrations supramolecular aggregates. The critical micellar concentration, i.e., the concentration at which monomers start to form aggregates, can be estimated to be in the nM range or lower [123, 124]. Thus the concentration of LPS monomers are extremely low and the formed aggregates play a major role in the activation of a number of processes [50]. Furthermore, it has been shown that only LPS aggregates are biologically active and not the monomers [51]. The formed supramolecular structures, cubic or lamellar structure, depend on the polar to apolar surface area ratio, and determine the ability of LPS to be active or inactive [43].

The solution structure of the two polymyxins (PMB and PME) were studied by Pristovšek and Kidrič (1999) [125] using homo-nuclear magnetic resonance (NMR) results combined to a molecular modelling study. It came out that the free peptides exist in an equilibrium of fast exchanging conformations; only local conformational preferences can be deduced from NMR data and NOE restrained structure calculations, namely a distorted type II' *beta*-turn extending from residues 5-8 and/or a gamma-turn in residue 10. Using photon correlation spectroscopy, Wiese (2001) [126] observed that above peptide concentrations of 10 mM peptide aggregation may occur.

As mentioned above, lipopolysaccharide are highly negatively charged molecules. Peptide that act as antimicrobial agents have in a first step to solubilise and lyse the bacterial cell membrane, but they have also to neutralise the LPS by peptide association [23]. Therefore, peptides with high affinities for LPS have to be developed with the ability to bind and thus neutralise free endotoxin molecules. Biomolecules bearing these properties are e.g. the bactericidal/permeability-increasing protein (BPI), cathelidins CAP18), *Limulus* antilipopolysaccharide factor (LALF), cyclic peptides derived from the LPS-binding domain [127], and polymyxin [23, 27, 127].

In order to induce a strong interaction of the peptide with LPS, most relevant peptides bear positive charges. Under physiological conditions, PMB bears 5 positive charges.

Thomas and co-workers (1999) [128] used surface plasmon resonance studies to elucidate the endotoxin-neutralising activity of PMB. Based on their data they tried to discriminate between the 3 possible ways by which PMB can act as a detoxification agent: (i) by altering the organization of the endotoxin in the lamellar phase, (ii) by coating the LPS lamellar phase, and/or (iii) by solubilizing and removing it from the LPS assembly. The SPR (surface plasmon reso-

nance) studies presented by Thomas *et al.* showed clearly that PMB is very effective in solubilizing endotoxin from its assembly. On the contrary, a number of peptides that interact with the endotoxin fail to do so. This is in accordance to the solubilisation effect that is detected in calorimetric experiments (Fig. 4). The phase transition of LPS Re, which represents the temperature induced transition from a gel phase (with acyl chains adopting more or less trans isomers) to a liquid-crystalline phase (acyl chains with increased amounts of trans-gauche isomers) is observed at ca. 32 °C. The presence of PMB induces a destabilisation of the gel phase, indicated by a shift of the heat capacity curve to lower temperature [129-131]. Furthermore, the phase transition enthalpy decreases dramatically as a function of the presence of PMB (Figures 5 and 6). This is an indication that the hydrophobic acyl chain of PMB penetrates and intercalates into the LPS aggregates. The aggregates are fluidised, more precisely, the LPS hydrocarbon chains adopt a higher amount of trans-gauche isomers, which is important for the physiological activity. A PMB-induced acyl chain fluidisation effect can also be demonstrated using infrared spectroscopy [132]. Tsubery *et al.* [133-135] came to a similar conclusion and demonstrated also the significant role of the hydrophobic segment of a derivative denoted as PMBN (PMB nonapeptide) in promoting biological activity.

Stopped-flow experiments performed by Thomas *et al.* [128, 136] also explicitly showed that the bi-molecular association between PMB and LPS is followed by the insertion of the hydrophobic parts of the antibiotic into the apolar milieu of the endotoxin lamellar phase which, as expected, is described well by a unimolecular reaction (see also [137]). Beyond electrostatic interactions between the peptide and the lipid the impact of ionic interactions have to be considered [138].

Furthermore, Tsubery *et al.* (2000) [135] showed that the functional association of PMB with bacterial lipopolysaccharide is stereospecific. Their results suggest that whereas the binding of the two enantiomeric PMB peptides to *E. coli* and to *E. coli* LPS is rather similar, functional association with the bacterial cell is stereospecific.

Removing the acyl chain of PMB, by which its derivative PMBN is formed. PMBN shows different interactions with LPS molecules (e.g. Fig. 4). Again, due to electrostatic interactions between PMBN and the lipopolysaccharide, the gel phase is destabilised. However, the decrease of the phase transition enthalpy is much lower, because of the absence of the acyl chain of the peptide (Fig. 5).

The interaction mechanism of PMB with endotoxins depend also on the phase state of the LPS [139, 140]. Brandenburg *et al.* (2005) [129] investigated the interaction between various endotoxins (from free lipid A to various LPS chemotypes with different sugar chain lengths) and PMB and PMBN by isothermal titration calorimetry as a function of temperature (20 – 50 °C). The results show a strong dependence of the titration curves on the phase state of the endotoxins. In the gel phase an endothermic reaction is observed, for which the driving force is an entropically driven endotoxin-polymyxin interaction, due to disruption of the ordered water structure and cation assembly in the lipid A backbone and adjacent molecules. In the liquid crystalline phase an exo-

thermic reaction takes place, which is mainly due to the strong electrostatic interaction of the polymyxins with the negative charges of the endotoxins, i.e., the entropic change is much lower than in the gel phase. For endotoxins with short sugar chains (lipid A, LPS Re, LPS Rc) the stoichiometry of the polymyxin binding corresponds to pure charge neutralization; for the compounds with longer sugar chains (LPS Ra, LPS S-form) this is no longer valid. This can be related to the lower susceptibility of the corresponding bacterial strains to antibiotics.

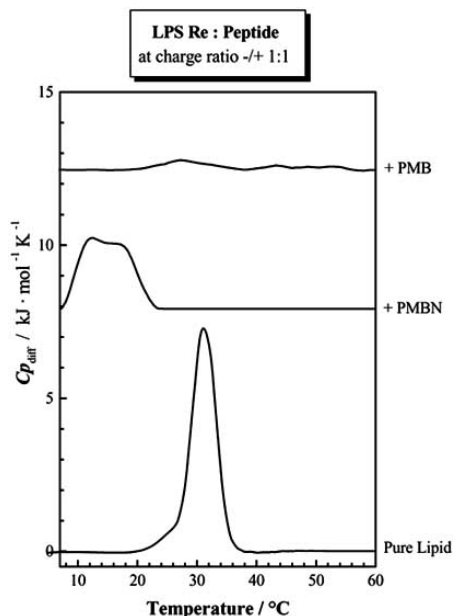


Fig. (4). Heat capacity curves of LPS Re at a 1:1 +/- charge ratio (corresponding to a molar ratio LPS Re : peptide 1:0.8) for PMB and PMBN.

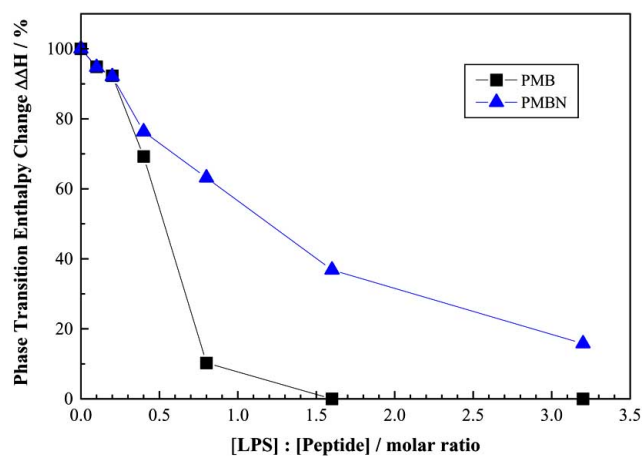


Fig. (5). Change in the phase transition enthalpy (expressed in %) as assessed by differential scanning calorimetry for LPS Re to peptide molar ratio for PMB and PMBN.

Two years later the same group [130], additionally were interested in the now known phenomenon of PMB resistance of bacteria, which is attributed to structural changes in the LPS moiety of the respective bacteria. They performed a thermodynamic and biophysical analysis to get insights into

the mechanisms of various LPS/PMB interactions by comparing LPS from sensitive and resistant strains. Their binding experiments based on isothermal titration calorimetry [139-141] revealed considerable differences of PMB binding to sensitive and resistant LPS. For sensitive LPS the endothermic enthalpy change in the gel phase of the hydrocarbon chains converts into an exothermic reaction in the liquid crystalline phase. In contrast, for resistant LPS the binding enthalpy change remains endothermic in both phases.

Furthermore, differences in the PMB concentration needed for LPS aggregate solubilisation was observed whether the strains were sensitive or resistant (Fig. 6) [130, 131]. The Zeta-potential (describing the electrokinetic potential) of the different LPS systems is somewhat different, and the presence of the peptide induces a strong reduction of its value (Fig. 7). This reduction in Zeta-potential indicates a strong interaction between the lipid and the peptides (PMB and PMBN).

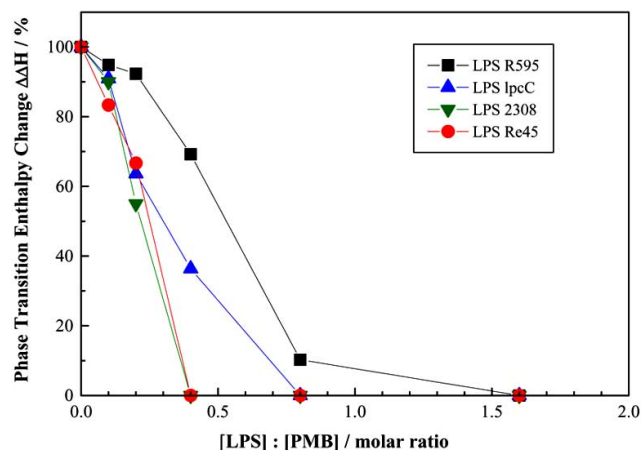


Fig. (6). Change in the phase transition enthalpy (expressed in %) as assessed by differential scanning calorimetry for different LPS chemotypes with increasing Lipid to PMB molar ratio (for more details see [129, 130]).

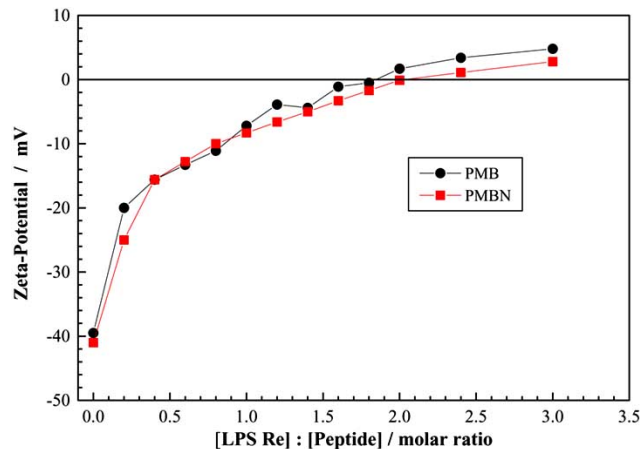


Fig. (7). Charge compensation upon LPS to PMB interaction as investigated by Zeta-potential measurements.

To understand the differences between the resistant and sensitive bacteria strains upon the interaction with PMB, it is important to look at the molecular level of the interaction,

namely between the interaction of the negatively charged phosphate groups and the positively charged amino acid residues of the peptide. Such information can be derived from infrared experiments (Fig. 8). For LPS R45 the interactions between the lipid and the peptide are weaker compared to the interaction with LPS R595. This can be deduced from the changes of the band shapes of the phosphate vibrations (Fig. 8). As infrared data show, these differences towards the PMB binding can be explained by steric changes in the headgroup region of the respective LPS.

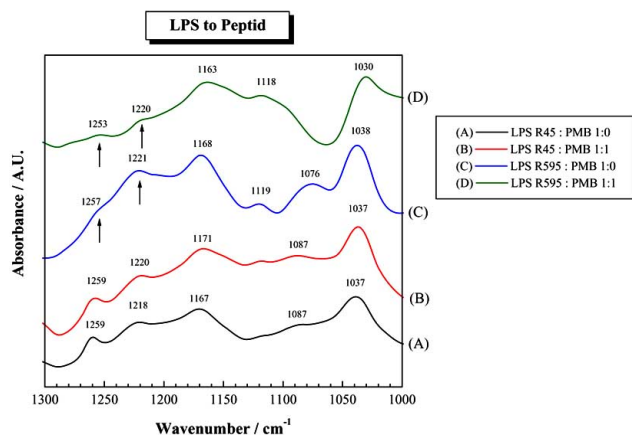


Fig. (8). Infrared spectra of LPS R45 and LPS R595 in the region of phosphate and sugar vibrations in the absence and presence of PMB (at 1:1 molar ratio). The corresponding bacterial strains are PMB-resistant and -sensitive, respectively.

The solution structure of PMB interacting with lipid A [125, 142] has been resolved, and the structure is such, that the peptide ring covers the polar surface area of the lipid A moiety. As mentioned above, the cyclic moiety of free PMB in solution is characterised by a type II' beta-turn centered at D-Phe⁵, and a gamma-turn at Thr⁹ with no transannular H-bonds [23]. These features are preserved in the LPS-PMB associate. In this complex the carbonyl groups form a polar, more or less flat surface on one face of the cyclic portion which overlies the glycosidic hydrophilic backbone of lipid A, and the linear part, which bears the peptide acyl chain oriented parallel to the long axis of lipid A. The gamma-amino groups of pairs of the Dab residues (Dab³/Dab⁴ and Dab⁷/Dab⁸) form bidentate ionic hydrogen bonds with the lipid A phosphates, which are separated by approximately 1.4 nm (for more details see David 2001 [23]).

Thus, the first step in the interaction of LPS with PMB is driven mainly by electrostatic interactions between the negatives charges of the lipopolysaccharide molecules of the outer membrane of Gram-negative bacteria and the five positive charges of the peptide. Gutschmann and colleagues [143] proposed that the interaction leads to a strong increase of the PMB concentration at the membrane surface. When the concentration of the peptide in the near vicinity of the outer LPS membrane reaches a certain concentration, which is similar to the concentration necessary for the observation of PMB self-association and thus the formation of PMB micelles, transient lesions can be observed in the LPS membrane (Fig. 9). This result is derived from conductance experiments us-

ing asymmetric planar membranes (for more details see Wiese *et al.* 1998 [144]). The lesion sizes that are detected depend strongly on the chemical structure of the LPS. A high number of effective negative charges of the lipopolysaccharide (e.g. LPS R595 from *Salmonella minnesota*) lead to an increasing number of PMB molecules bound to the membrane, which finally induce lesions with a diameter of more than 2 nm. Gutschmann and co-workers have detected by means of atomic force microscopy, the formed lesions and were able to show that these lesions are not stable [143]. The investigation of resistant bacterial strains [145] such as *Proteus mirabilis*, the LPS of which is characterised by a reduced effective net charge due to the substitution by L-Arap4N, shows that PMB is also able to induce the formation of lesions in planar membranes, however, with lesion diameters less than 1 nm [146]. Considering the diameter of PMB molecules being about 1.25 nm according to film balance measurements, it is likely that the lesions formed in resistant bacterial strains are too small for the promoted uptake of PMB. For bacterial strains like *S. minnesota* where PMB-induced lesions of more than 2 nm in diameter are formed, a self-promoted peptide uptake is possible.

Gaugelavičius *et al.* (2000) [147] investigated the PMB induced permeabilities of the outer and cytoplasmic membrane of *Escherichia coli* by monitoring the fluxes of tetraphenylphosphonium, phenyldicarbaundecaborane, and K⁺ and H⁺ ions. The major findings of this work was summarised as follow: (i) the outer membrane-permeabilizing action of PMB has to be dissected from its depolarizing effect on the cytoplasmic membrane, (ii) the PMB-induced ion-permeable pores in bacterial envelope were registered, and (iii) the pore formation and depolarization of the cytoplasmic membrane are not obligatory for the bactericidal action of PMB and dissipation of the K⁺ gradient on the cytoplasmic membrane.

Gaugelavičius *et al.* (2000) [147] also observed a reduction of the permeabilizing effect of the outer membrane by PMB by increasing the Mg²⁺ concentration.

Wiese *et al.* (1998) [144] used electrical measurements for the investigation of the time-dependent current fluctuations of reconstituted Gram-negative outer membrane bilayers composed of LPS molecules. The presence of PMB on the LPS side of the bilayer showed an increase of the current fluctuations, which is explained by the PMB-induced formation of defects and lesions in the membrane. Again, they observed that the size of the formed lesions are LPS-dependent; small lesions are detected for resistant, whereas larger lesions are observed for PMB-sensitive bacterial strains. The data presented by Wiese *et al.* (1998) [144], but also Schröder *et al.* (1992) [148] gave indications that for the initiation of the formation of single membrane lesions a threshold concentration of the drug at locally restricted areas at the membrane surface is required and that the lipid molecules of the bilayer matrix are involved in the formation of the lesions. These observations of PMB-induced membrane solubilisation have some aspects that resemble the solubilisation processes as observed by detergents [149]. The exact mechanistic aspects by which membranes are solubilised/lysed by peptides are quite different and still of great interest [150-154].

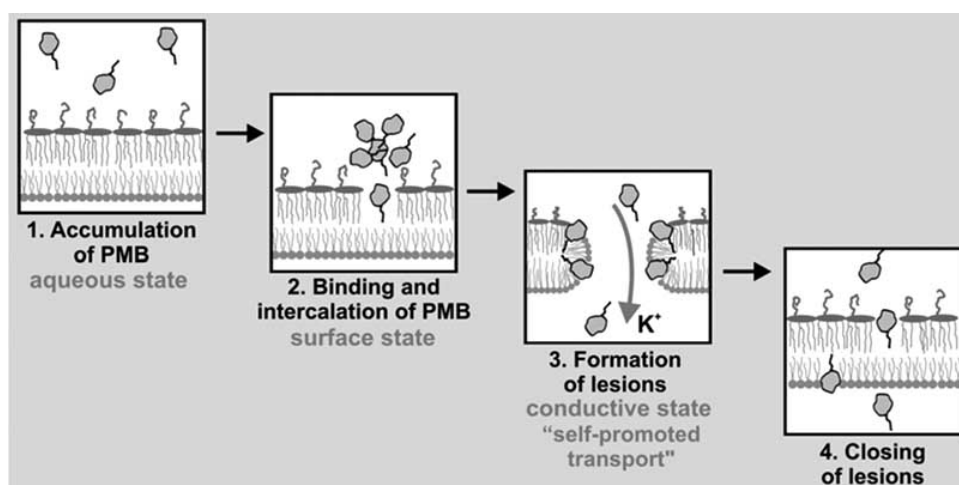


Fig. (9). Schematic representation of the interaction of PMB with the endotoxin (LPS) layer at the outer membrane surface. First: electrostatic interaction between negatively charged LPS molecules and positively charged PMB, second: binding and intercalation of the hydrophobic acyl chain of PMB between LPS molecules and disrupting the membrane structure, respectively, leading to pores with a sufficient diameter to allow the PMB molecules to act intracellularly (see [144]).

Thirty years ago, Cooperstock (1977) [22] discussed a mechanism by which the cationic molecules of polymyxin B and colistin compete and displace divalent Ca^{2+} and Mg^{2+} cations, which normally stabilize the lipopolysaccharide molecule of the outer membrane of Gram-negative bacteria. Both of these compounds are bactericidal, targeting the bacterial cell wall, disrupting membrane permeability, and ultimately leading to cell death. The polymyxins exert their bactericidal activity by binding to the bacterial cell membrane and disrupting its permeability, which results in leakage of intracellular components. They also have antiendotoxin activity [22].

It is known that PMB, which binds to and neutralizes the toxic residue of bacterial lipopolysaccharide, greatly amplifies cellular responses mediated by the P2X7 receptor, which is a membrane receptor for extracellular ATP. P2X7 is highly expressed in dendritic cells, macrophages and microglia where it mediates pro-inflammatory responses. But up to now, the involved molecular mechanisms are quite unclear. In order to elucidate the molecular mechanism, Ferrari *et al.* (2007) [155] used human macrophages and HEK293 cells that stably expressed the human P2X7 receptor (HEK293-hP2X7) and investigated the effect of PMB and PMBN. The observed differences between the two peptides were assessed by monitoring the changes in the nucleotide-induced cytoplasmic free Ca^{2+} concentration, plasma membrane permeability, lactate dehydrogenase activity, and cell morphology changes.

The main results were that in contrast to PMB, the PMBN was unable to potentiate i) the ATP-induced Ca^{2+} increase, ii) pore formation and consequently ATP-mediated plasma membrane permeabilization; and iii) show ATP-dependent cytotoxicity. Additionally, in contrast to PMB, PMBN did not affect aggregation of the P2X7 receptor subunits and it did not potentiate P2X7-dependent cell fusion. Ferrari *et al.* (2007) [155] concluded that the PMB effects were due to the presence of its N-terminal fatty amino acid 6-methylheptanoic/ octanoic-Dab residue, the deletion of this residue abolished the PMB-dependent modulation of

ATP-triggered responses. These findings have a certain significance in the search for allosteric modulators of the P2X7 receptor.

The role of membrane permeabilisation and disruption in the mechanism of action of some polymyxin analogues against Gram-negative organisms is discussed controversially. The effects of PMB and PMBN on *Escherichia coli* envelopes should correlate, but previous studies suggest that PMBN has a different mode of action. Sahalan and Dixon (2008) [156] have recently reassessed the biochemical techniques used previously for the investigation of polymyxin and derivatives thereof with LPS and has shown that, in contrast to previous studies, PMBN readily releases periplasmic proteins and lipopolysaccharide from treated *E. coli* at sub-inhibitory concentrations under normal physiological buffer conditions. These authors concluded that, when tested with the appropriate methodology, PMBN closely correlates with the early effects of PMB on the cell envelope of *E. coli* and showing that their investigation is now consistent with the accepted interactions of membrane-active agents against Gram-negative cells.

CONCLUSIONS

The polymyxins belong to the best investigated peptides/antibiotics used in the fight against infectious diseases. Due to newer findings, they may play a significant role in treatment of bacterial sepsis on critical care stations induced by Gram-negative bacteria. Furthermore, the data reviewed here representing a complete characterization of the medical, pharmacological, and biophysical effects of the polymyxins on bacteria and on isolated endotoxins can be taken as calibration standard for newly developed antimicrobial peptides, such as those derived from the binding domains of LPS-binding proteins like *Limulus* anti-LPS factor and lactoferrin.

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