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Bacterial cell wall compounds as promising targets of antimicrobial agents I. Antimicrobial peptides and lipopolyamines

Guillermo Martinez de Tejada¹, Susana Sánchez-Gómez¹, Ina Kowalski², Yani Kaconis², Jörg Andrä², Tobias Schürholz³, Mathias Hornef⁴, Aline Dupont⁴, Patrick Garidel⁵, Thomas Gutschmann², Sunil A. David⁶, and Klaus Brandenburg²

¹University of Navarra, Dept. of Microbiology, Irunlarrea 1, E-31008 Pamplona, Spain

²Forschungszentrum Borstel, Div. of Biophysics, Parkallee 10, D-23845 Borstel, Germany

³Universitätsklinikum Aachen, Pauwelsstr. 30, D-52074 Aachen, Germany

⁴Medizinische Hochschule Hannover, Carl-Neuberg-Str. 1, D-30625 Hannover, Germany

⁵Martin-Luther-Universität Halle-Wittenberg, von-Danckelmann-Platz 4, D-06120 Halle, Germany

⁶University of Kansas, 4070 Malott Hall, Lawrence, Kansas, USA

Abstract

The first barrier that an antimicrobial agent must overcome when interacting with its target is the microbial cell wall. In the case of Gram-negative bacteria, additional to the cytoplasmic membrane and the peptidoglycan layer, an outer membrane (OM) is the outermost barrier. The OM has an asymmetric distribution of the lipids with phospholipids and lipopolysaccharide (LPS) located in the inner and outer leaflets, respectively. In contrast, Gram-positive bacteria lack OM and possess a much thicker peptidoglycan layer compared to their Gram-negative counterparts. An additional class of amphiphiles exist in Gram-positives, the lipoteichoic acids (LTA), which may represent important structural components. These long molecules cross-bridge the entire cell envelope with their lipid component inserting into the outer leaflet of the cytoplasmic membrane and the teichoic acid portion penetrating into the peptidoglycan layer. Furthermore, both classes of bacteria have other important amphiphiles, such as lipoproteins, whose importance has become evident only recently.

It is not known yet whether any of these amphiphilic components are able to stimulate the immune system under physiological conditions as constituents of intact bacteria. However, all of them have a very high pro-inflammatory activity when released from the cell. Such a release may take place through the interaction with the immune system, or with antibiotics (particularly with those targeting cell wall components), or simply by the bacterial division. Therefore, a given antimicrobial agent must ideally have a double character, namely, it must overcome the bacterial cell wall barrier, without inducing the liberation of the pro-inflammatory amphiphiles. Here, new data are presented which describe the development and use of membrane-active antimicrobial agents, in particular antimicrobial peptides (AMPs) and lipopolyamines. In this way, essential progress was achieved, in particular with respect to the inhibition of deleterious consequences of bacterial infections such as severe sepsis and septic shock.

Keywords

Antimicrobial peptides; endotoxin shock; lipopolyamines; lipopolysaccharides; tumor-necrosis-factor α .

Introduction

Bacterial infections are still one of the major threats to human health worldwide. Bacterial pathogens frequently cause severe diseases not only as primary agents, but also subsequent to pathologies caused by other agents (superinfection such as ‘Spanish flu’, [1]). This fact is, due, at least in part, to the increasing occurrence of bacterial resistance to antibiotics. In turn, the spread of antibiotic resistant clones is greatly enhanced by the improper use of antibiotics in daily medicinal practise and by the massive application of antibiotics in animal husbandry [2]. Furthermore, in the last decades only two antibiotic classes with novel mechanism of action have been marketed, and none of them are effective against Gram-negative bacteria.

On the other hand, the antimicrobial activity of a given antibiotic does not correlate with its ability to inactivate bacterial pathogenicity factors (PF) and interaction of some antibiotics with their bacterial targets *in vivo* even enhance the release of PF into the extracellular milieu. This frequently results in bacterial sepsis [8], a pathology that is the leading cause of death in the critical care units of hospitals worldwide.

The cell wall is the first barrier that an antimicrobial agent must counteract [12]. In contrast to Gram positive bacteria, Gram-negative organisms have an additional membrane, the so called outer membrane (OM), that lies over and covers both the cytoplasmic membrane and the peptidoglycan layer. The OM has an asymmetric distribution of the lipids with phospholipids and lipopolysaccharide (LPS) located in the inner and outer leaflets, respectively [13].

Gram-positive organisms lack OM and possess a much thicker peptidoglycan layer compared to their Gram-negative counterparts [12]. The additional class of amphiphiles in Gram-positives, the lipoteichoic acids (LTA), representing important structural components [14], cross-bridge the entire cell envelope with their lipid component inserting into the outer leaflet of the cytoplasmic membrane and the teichoic acid portion penetrating into the peptidoglycan layer. The importance of another class of amphiphiles, the lipoproteins, has become evident only recently [15]. Summarized, the main PF of Gram-negative bacteria is the endotoxin (LPS, lipopolysaccharide), whereas lipoproteins (LP) rank among the most important PF in Gram-positive. Therefore, an ideal antibiotic should combine a high antimicrobial activity with a potent ability to bind and neutralize PF.

A promising approach to develop new effective drugs since the 1990’s is the use of AMPs derived from binding domains of host defence proteins such as lactoferrin, NK-lysin, and *Limulus* anti-LPS factor (LALF) [9], [10], [11]. Successive cycles of improvement rendered compounds endowed with considerable antimicrobial and anti-endotoxic activity. In addition, pathogens are frequently unable to develop resistance to these rapidly bactericidal agents [3]. For instance, some pathogens such as *Pseudomonas aeruginosa* remain sensitive only to polymyxins [4], although the systemic use of these AMPs is not widely accepted by clinicians due to their alleged nephro- and neurotoxicity [5]. An important feature of the majority of AMPs is their ability to bind to LPS and permeabilize the outer membrane (OM) of Gram-negative bacteria thus sensitizing bacterial cells to co-administered compounds [6]. In addition, the ability of AMPs to kill cells in stationary phase enables them to be bactericidal not only for planktonic cells but also for bacteria growing in biofilms [7]. Up to now, however, available peptides lack a sufficiently high therapeutical index, which is necessary to avoid detrimental side effects.

Whereas it is clear that amphiphilic components such as LPS and LTA provide important permeability barrier functions, it has not been demonstrated that these molecules stimulate the innate immune system as constituents of intact bacterial cell wall structures. However,

all of them are highly active when released from the cell and this occurs either by the action of the immune effector functions (such as complement-mediated lysis, opsonophagocytosis, etc.), or by antibiotics, or simply by cell division of the bacteria. The clinical significance of antibiotic-mediated release of these immunostimulatory and proinflammatory amphiphiles is of high relevance [16], [17]. It is therefore desirable that antimicrobial regimens not only exert bactericidal activity, but also either avoid release of proinflammatory cell wall-derived amphiphiles, or sequester and neutralize them even as they are being released as a consequence of microbial lysis and death.

We present data describing the development and use of dual-purpose membrane-active antimicrobial agents and LPS/LP sequestrants, focusing on AMPs and lipopolyamines. Our long-term goal is to evaluate these compounds either as single-agent antimicrobials or as adjunctive chemotherapeutic agents that may have value in combination with bactericidal antibiotics. These compounds have been shown to effectively neutralize LPS *in vitro* and to protect mice from a lethal endotoxic shock in an animal model of acute sepsis. Different aspects of the mechanisms of action in *in vitro* as well as *in vivo* systems are described in this paper.

Peptide-induced inhibition of inflammation reaction

Lactoferricin-derived peptides

It has been previously shown that acylation of a lactoferricin-derived AMP leads to an improvement both of the antimicrobial and LPS detoxification activities of the resultant compound [18].

To confirm and expand these observations, we used LF11, an 11-amino acid (FQWQRNIRKVR) peptide derived from human lactoferricin, as a template to design a library of peptides and lipopeptides as described elsewhere [19, 20]. To study the influence of acylation on the antimicrobial activity of the compounds we measured their minimum inhibitory concentration (MIC) on Gram-negative and Gram-positive bacterial pathogens grown under conventional (planktonic) culture conditions. In addition, we tested the ability of the peptides and their acylated derivatives to reduce the viability of biofilms formed by *Pseudomonas aeruginosa* PAO1 and/or to remove them. Results obtained with peptides P2-15, P2-27, P2-29 and P2-33 (see Table 1) exemplified to a large extent the behaviour observed with the entire library. In general, acylation neither increased the potency nor the spectrum of antimicrobial activity of the compounds since lipopeptides had equal or worse MIC than their parent molecules. These results show that sometimes acylation of a lactoferricin-derived peptide can be detrimental for its antimicrobial performance as opposed to previous observations with LF11 and its N-acyl derivative lauryl-LF11 [18]. In addition, our data confirm conclusions of Japelj and collaborators reporting that in synthetic lipopeptides the acyl chain represents an integral part of the molecular structure thus influencing the shape of the lipopeptide for optimal interaction with bacterial membrane [21]. Notably, the only organism that was more sensitive to the acylated than to the parent compounds was *Brucella abortus*, a pathogen endowed with a lipopolysaccharide and a cell envelope unconventional for a Gram-negative bacterium [22].

Testing the antimicrobial activity of compounds on biofilms instead of on planktonic cells heightened the differences between peptides and lipopeptides. Thus, whereas the former agents killed at least 95 % of cells and showed a significant biofilm-removing activity, most lipopeptides displayed a very modest performance under the same conditions. The low anti-biofilm activity of lipopeptides may be due to their higher affinity for alginate, a component of the extracellular matrix of *P. aeruginosa* biofilm that has been reported to bind hydrophobic compounds [23]. Additionally, it is possible that the higher molecular weight

of the acylated compounds may hinder their penetration into the extracellular matrix of the biofilm.

To study if acylation improved the OM-permeabilizing activity of peptides, we measured the ability of all the compounds to act in synergy with novobiocin against *P. aeruginosa*. This hydrophobic antibiotic cannot reach its intracellular target, gyrase, due to its inability to cross an intact outer membrane. To quantify the synergistic effect, we determined the Fractional Inhibitory Concentration (FIC) index of the peptide-novobiocin combinations. The majority of authors consider a combination as synergistic when its FIC index is < 0.5 . As shown in Table 1, acylation systematically resulted in compounds devoid of the potent novobiocin-enhancing activity displayed by the parent peptides. In agreement with this observation, Vaara and Vaara demonstrated that OM-permeabilizing activity does not depend on acylation since PMBN, a deacylated derivative of PMB, is one of the most potent permeabilizers known to date [24]. The exception to this behaviour was peptide P2-29, the compound rendering the lowest MIC on the test strain (31.25 $\mu\text{g/mL}$). Lack of synergistic activity of P2-29 could be due to the fact that its lethal (bactericidal) activity is so great that it is not possible to observe non-lethal (permeabilization) activity, as it has been reported previously [20].

In light of our results, one could conclude that acylation of peptides may be of minor importance since it appears to lead to compounds lacking any meaningful biological property. To the contrary, our data demonstrate that the ability to bind LPS *in vivo* and to protect animals from a lethal endotoxic shock is strongly dependent on acylation (see Table 1). In all the cases, protection was associated with a very significant drop in TNF α serum levels 60 min after inoculation of the lipopeptide (data not shown). These observations are in good correlation with those mentioned above by Andr  and collaborators [18] and by Tsubery and collaborators [25] reporting that lipopeptides display a much higher LPS neutralizing activity compared to their non-acylated counterparts. The degree of protection conferred by the lipopeptides was very high and homogenous with very few exceptions (Table 1 and our unpublished results). Therefore, it was not possible to establish relationships between antiendotoxic activity and the presence of a particular amino acid sequence or acyl groups in the compounds. Our results demonstrate that acylation is sufficient in most of the cases to turn a peptide with poor affinity towards LPS into a compound endowed with potent anti-endotoxic activity *in vivo*.

Peptides based on *Limulus* anti-LPS factor and derivatives

For the development of suitable compounds to neutralize bacterial endotoxins a peptide library was constructed which were originally based on the binding region of the *Limulus* anti-LPS factor (LALF) [26]. The LALF was crystallized and the LPS-binding domain was identified [27]. Ried et al [28] were the first authors reporting synthesis of peptides based on this domain. They found that the complete binding sequence cLALF22 (amino acid sequence 31–52 of LALF: GCHYRIKPTFRRLKWKYKGFWCG) had the highest ability to bind to the lipid A part of LPS, its ‘endotoxic principle’, whereas the shortened analogs down to cLALF10 (LALF sequence 38–45, GCTFRRLKKCG) were much less active. Andr  et al performed biophysical studies of the interaction of LALF (in recombinant form called ENP = endotoxin-neutralizing protein) and part structures with endotoxins [9], [29], [30], and found considerable inhibition of the biological activity of LPS at [ENP]:[LPS] 20 to 200:1 molar. Parallel to this, data on the aggregate supramolecular structure of LPS, performed with synchrotron radiation small-angle X-ray scattering (SAXS), were indicative of a change of the lipid A cubic structure into a more lamellar one. Importantly, determination of the surface potential of LPS, the Zeta potential, which is a measure of the accessible negative charges, showed an increase from -70 mV to positive values for lipid A

and LPS Re. LALF-derived cyclic peptides were analysed in subsequent studies [31], [32], starting with the compound with the complete LPS-binding domain (cLALF22) and various shortened analogues. In all these studies the data consistently showed a change of the endotoxin aggregate structure from preferentially cubic into a multilamellar one, as well as a change of the morphologies of the LPS Re aggregates, as evidenced by freeze-fracture electron microscopy, from 'open egg-shells' (i.e., spherical particles in the range 100 to 200 nm), into large stacks of some 1000 nm. The latter structures corresponded to the multilamellae in the SAXS experiment with periodicities of 6.3 to 7.5 nm. With isothermal titration calorimetry (ITC) the binding of the peptides to LPS in all cases was characterized as an exothermic process, driven by the Coulomb interaction of the positive charges of peptides with the negative charges of the endotoxins.

The terminal part of the shrimp anti-LPS-factor, a synthetic peptide with 24 AA, cyclic and linear with a sequence Ac- ECKFTVKPYLKRFRQVYYKGRMWCP, was analysed by Pan et al [31]. Pre-treatment of mice with the cyclic compound led to a considerable enhancement of survival of mice which had been infected with $1.28 \cdot 10^6$ cfu from *Pseudomonas aeruginosa*, and the peptides were able to decrease the bacteria-induced production of TNF α in the animals. Unfortunately, the peptides exhibited at a concentration of already 2 μ g/ml significant cytotoxicity in HeLa, MCF-7, and HT1080 cell lines, as measured by the MTT test.

Due to the inability of the above described peptides to get a complete anti-septic action, we have designed and constructed a new series of 19' mer peptides. The biophysical analysis of peptide 19-2 (sequence see Table 2) exhibited strong endotoxin binding, as deduced from ITC experiments, resulting in a nearly complete blocking of the LPS-induced TNF α production of human MNC at [Pep]:[LPS] 100:1 weight% [32]. A slightly shortened variant exhibited much less anti-endotoxic activity, but considerably higher antibacterial activity. This illustrates that the two parameters, antibacterial and anti-endotoxin activity, not necessarily correlate.

The systematic study of various peptides differing in their amino acid chain lengths from 9 over 12 and 17 to 19, showed that the basic sequence, as given by Pep9, is already able to confer a significant inhibition of the LPS-induced cytokine production (Fig. 1). However, relatively high concentrations of the peptides were still necessary to inhibit cytokine production and we found that we could improve peptide's activity in this respect by prolonging the peptide length with suitable amino acids. As can be seen in the Figures 1–2, the increase in the AA acid chain lengths must be adapted to the physico-chemistry of the lipid A part. To achieve this, the N-terminal side of the peptide must be composed essentially of polar and basic AA, the C-terminal must be more hydrophobic. This alone, however, is not enough, the exact number and type and sequence of AA is important, as deduced from the comparison of the peptides with different chain lengths (Fig. 1) as well as the comparison of Pep19-5, -6, and -7 (Fig. 2).

The importance of an exact length and composition of the AA to neutralize LPS is illustrated by the results shown in Table 3, in which the action of two peptides with 17 AA is compared with those of Pep19-2 in a mouse model of sepsis, yielding only protection for the longer peptide.

Further improvements were obtained by modifications of the sequences (AA sequences see Table 2), which led to a nearly complete inhibition of the LPS-induced cytokine secretion at a [Pep]:[LPS] 3:1 molar ratio [33]. This is particularly evident in the case of the peptide Pep19-2.5, our lead structure, which could protect animals in the mouse model of sepsis already at a [Pep]:[LPS] ratio of 50:1 weight % (25 ng LPS and 1250 ng peptide). Also,

peptides Pep19-2.2 and Pep19-12.1 were very effective in protecting animals in the animal model of sepsis at peptide concentrations far below their threshold of cytotoxicity (Fig. 3). These investigations were performed with a rough mutant LPS Ra from *S. minnesota* strain R60. To assure that such strong inhibition activity takes also place for naturally occurring heterogeneous smooth mutant LPS, the inhibition of the cytokine secretion induced by LPS from *E. coli* O8:K27 was monitored by using the lead structure Pep19-2.5 (Fig. 4). As can be seen, also for this endotoxin a considerable reduction of the LPS-induced concentration of TNF α takes place already at [Pep]:[LPS] 3:1 weight %.

Since it was reported in literature, that peptides in a cyclic form, made by connecting two cysteins via disulfide bridges, had higher cytokine inhibitory activity than the corresponding linear peptides [34], we have constructed peptide Pep19-12.1 besides in its linear also in a cyclic form by bridging the second Cys at the N-terminal with the third Cys as seen from the C-terminal side (see Tab. 2). The comparison of the two peptides to inhibit the LPS-induced cytokine reaction showed unequivocally (Fig. 5) that the cyclic form, although still active, had significantly less ability to suppress the inflammation reaction.

Finally, for therapeutical use it would be of essential importance that some decrease of the inflammation reaction takes place also, when the peptide is administered in a time-delayed mode. Therefore, the peptide Pep19-2.2 was added at times 0 up to 3 h after the LPS administration (whole incubation time 4 h). As can be deduced from Fig. 6, there is still a considerable inhibition of the TNF α production even when the peptide is added 3 h after LPS addition, despite the fact that the action of the peptide can take place only in the remaining stimulation time of 1 h.

As mechanism of action for the ability of the peptides to neutralize endotoxins, the change of lipid A / LPS cubic aggregate into a multilamellar structure was proposed, connected with an extremely low saturation value of binding at [peptide]:[LPS] = 0.3 M/M, corresponding to the binding saturation of 3 peptides with 10 LPS molecules [34]. Thus, human binding proteins such as LBP and CD14 are no more able to bind to the LPS epitopes and thus cannot initiate the inflammation reaction. Furthermore, the ability of the peptides to act even after LPS administration is indicative of a membrane process of the interaction. This is presently under investigation.

Neutralization of endotoxin by lipopolyamines

Recognition that membrane-active antimicrobial compounds may also possess LPS-sequestering and -neutralizing activities was first identified with polymyxin B [36]. As described above, a variety of cationic, amphipathic peptides share these properties, as was evident with melittin, a basic, amphipathic peptide constituent of bee venom [37], as well as structurally unrelated peptides such as gramicidin S, tyrocidin, efrapeptin as well as *de novo* designed peptides [38],[39],[40]. Hypothesizing that polycationicity and amphipathicity are necessary and sufficient requirements for bacterial membrane perturbation and for LPS sequestration, we turned our attention to small molecules embodying the above-mentioned physical properties. Upon finding that *bis*-arg Gemini compounds indeed displayed microbicidal and LPS-sequestering [41] activities, and that DOSPER, a model lipopolyamine afforded protection in murine models of both purified LPS- and antibiotic-induced LPS release models [42] led us eventually to a careful examination of the lipopolyamine class of compounds typified by *N*-acyl or *N*-alkyl spermine or homospermine [43], [44], [45],[46], [47], [48], [49],[50].

In examining a homologous series of mono- and *bis*-acyl polyamines, it was noted that the carbon number (hydrophobicity) was a critical structural determinant of LPS-neutralizing

potency [50]; for the mono-acyl **4** series of compounds, there was a progressive increase in LPS-neutralizing potency (Fig. 7), while for the bis-acyl **8** series, the activity progressively decreased with acyl chains longer than dodecyl (C₁₂). It was of interest to probe possible correlations with MICs against *E. coli* versus *S. aureus*. The results shown in Fig. 8 indicate that against both organisms, a very similar structure-activity correlation is observed. Thus, for the **4** compounds, acyl chain lengths from C₁₂ to C₁₆ result in maximal antimicrobial efficacy against *S. aureus*, while maximal antibacterial effects are observed between C₁₂ to C₁₄ against *E. coli*, with the activity falling off at C₁₆, suggesting that the structural requisites for optimal interaction with the Gram-negative outer membrane are rather specific. For the **8** series, however, the converse is true with the short chain (C₈₋₁₁) analogues exhibiting maximal antibacterial effect (Fig. 9); the decline in activity in the higher homologues in the **8** series is ascribable to progressive loss of aqueous solubility. These structure-activity relationships of these compounds closely mirror that of LPS-induced TNF- α and nitric oxide (NO) production in murine macrophages.[46] The similarities of the **4** and **8** series between antimicrobial activity against Gram-negative bacteria on the one hand, and sequestration of LPS on the other, suggest that the antimicrobial activity is likely mediated via the interaction of these compounds with the outer membrane [50].

Our primary interest in examining these compounds, however, lay not so much in evaluating their intrinsic antimicrobial properties, but rather in understanding the mechanisms and structure-activity relationships underlying their putative membrane permeabilizing action, and in exploring the possibility of employing such compounds as adjuncts to conventional antimicrobial chemotherapy for purposes of sequestering endotoxin released as a consequence of Gram-negative bacterial lysis. We first investigated if the acylpolyamines would act on both the inner membrane (IM) and the outer membrane (OM) [50], [51], presumably as a consequence of nonspecific membranophilic effects as has been reported for a variety of cationic amphipathic peptides such as melittin [37], defensins [51], bactenecins [52] or, selectively perturb the OM in the manner of polymyxin B [53]. This is shown in Fig. 8, a direct linear relationship was observed between OM and IM permeabilizing activities [50]. Furthermore, these two events seem tightly coupled with near-identical kinetics even under conditions of high osmotic strength. It is to be noted that IM damage would necessarily require antecedent OM lysis.

Hypothesizing that compounds that sequester LPS could also bind to and inhibit LTA-induced cellular activation, we screened congeneric series of polyamine sulfonamides which we had previously shown effectively neutralized LPS both *in vitro* and in animal models of endotoxemia. We observed that these compounds indeed bind and neutralize LTA, as reflected by the inhibition of TLR2-mediated NF- κ B induction in reporter gene assays. Structure-activity studies showed a clear dependence of the acyl chain length on activity against LTA in compounds with spermine and homospermine scaffolds [54]. We then sought to examine possible correlations between the neutralizing potency toward LTA and antimicrobial activity in *S. aureus*. A linear relationship between LTA sequestration activity and antimicrobial activity for compounds with a spermine backbone was observed, while all compounds with a homospermine backbone were equally active against *S. aureus*, regardless of their neutralizing potency toward LTA [54]. These results suggest that the number of protonatable charges is a key determinant of the activity toward the membranes of Gram-positive bacteria.

The development of resistance to membrane-active antibiotics has been relatively slower than that to conventional antibiotics, and it is possible that compounds such as the acylpolyamines may be useful clinically, provided that they have an acceptable safety profile and margin of safety. A more detailed understanding of the mechanisms of

interactions of these compounds with LPS and LTA, as well as the Gram-negative and -positive bacterial cell surfaces, will be instructive and should allow the rational design of analogues which combine antiseptics and antibacterial properties.

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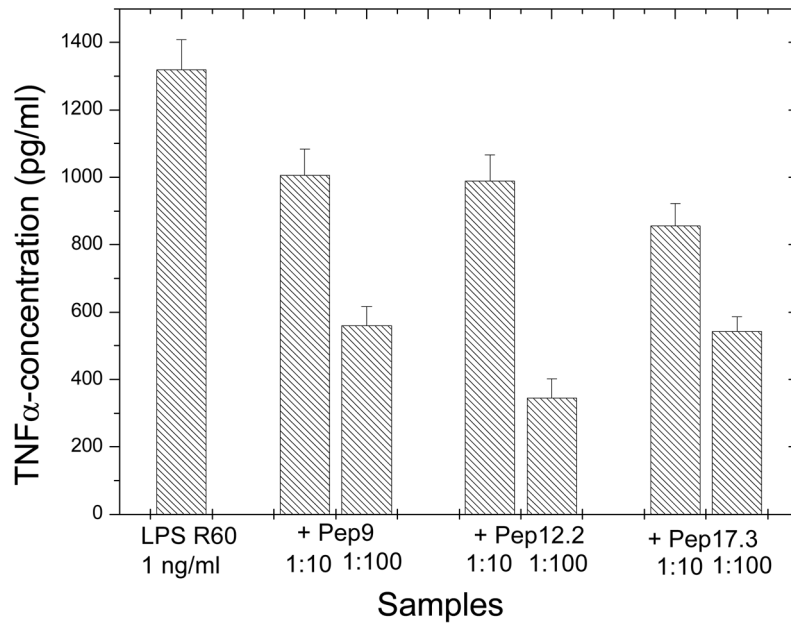


Figure 1. Inhibition of the LPS-induced production of tumor-necrosis-factor α in human mononuclear cells by peptides with different AA chain lengths at different [LPS]:[Pep] weight ratios.

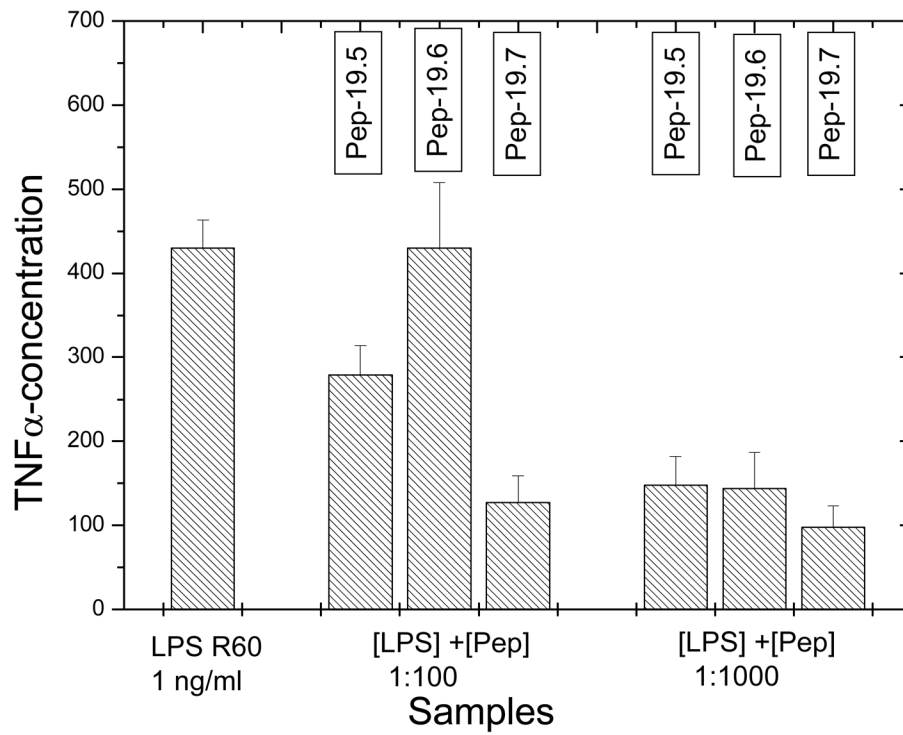


Figure 2. Inhibition of the LPS-induced production of tumor-necrosis-factor α in human mononuclear cells by peptides Pep19-5, Pep19-6, and Pep19-7 at different [LPS]:[Pep] weight ratios.

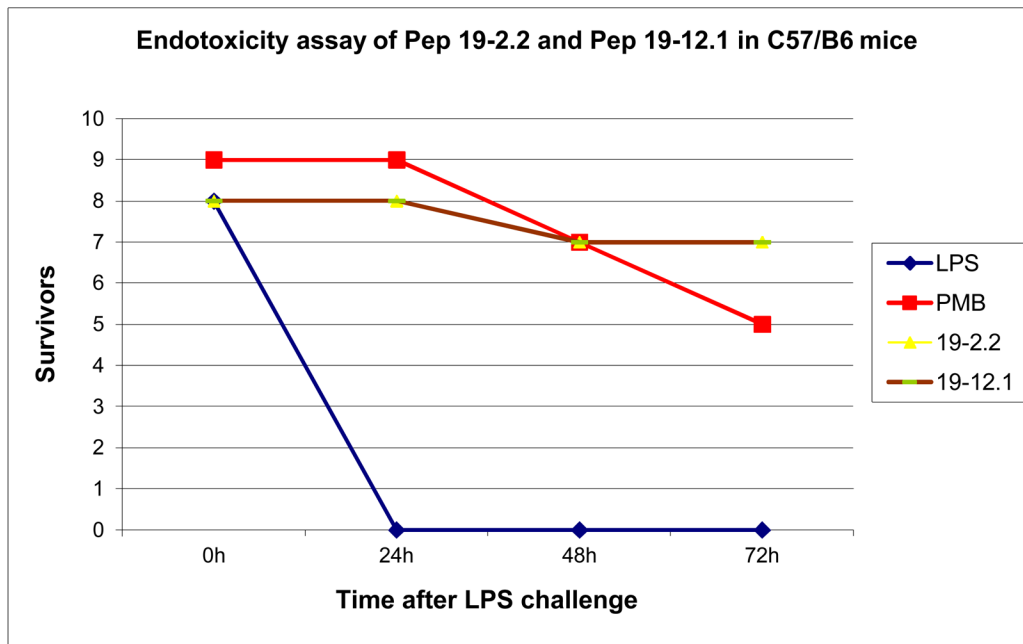


Figure 3. Survival of mice in the galactosamine model of endotoxin shock for Pep12-2 and Pep12.1 in comparison to the gold standard polymyxin B (PMB). LPS from *Pseudomonas aeruginosa* was administered i.p. at a concentration of, and the peptides were added at a concentration of

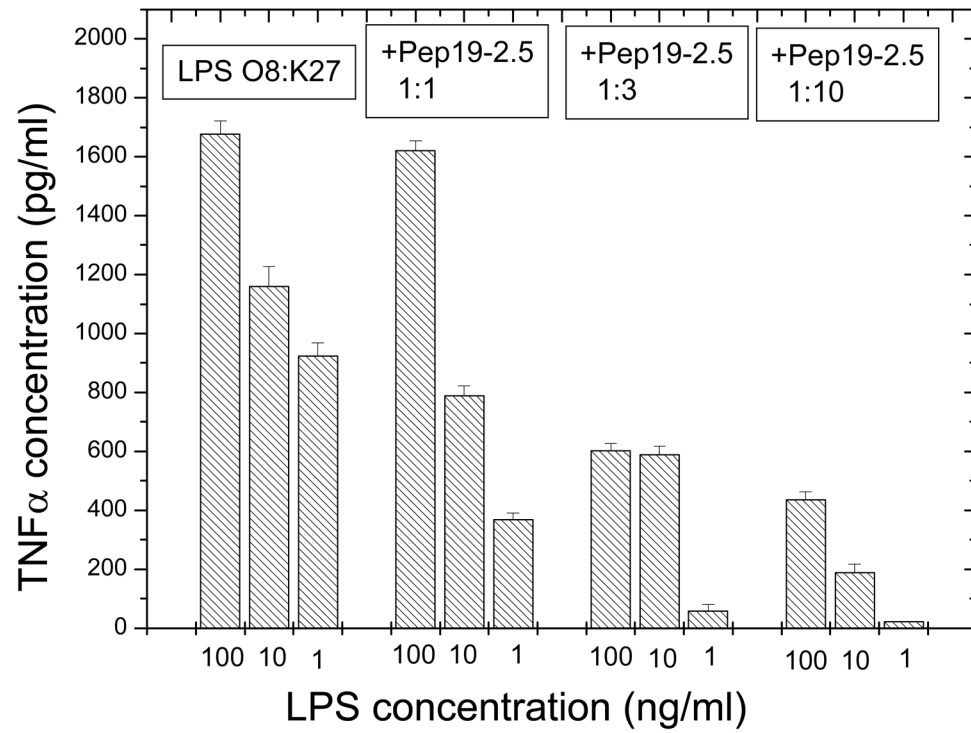


Figure 4. Inhibition of the tumor-necrosis-factor in human mononuclear cells, induced by LPS from *E.coli* O8:K27, by the lead peptide Pep19-2.5 at three different [LPS]:[Pep] weight ratios.

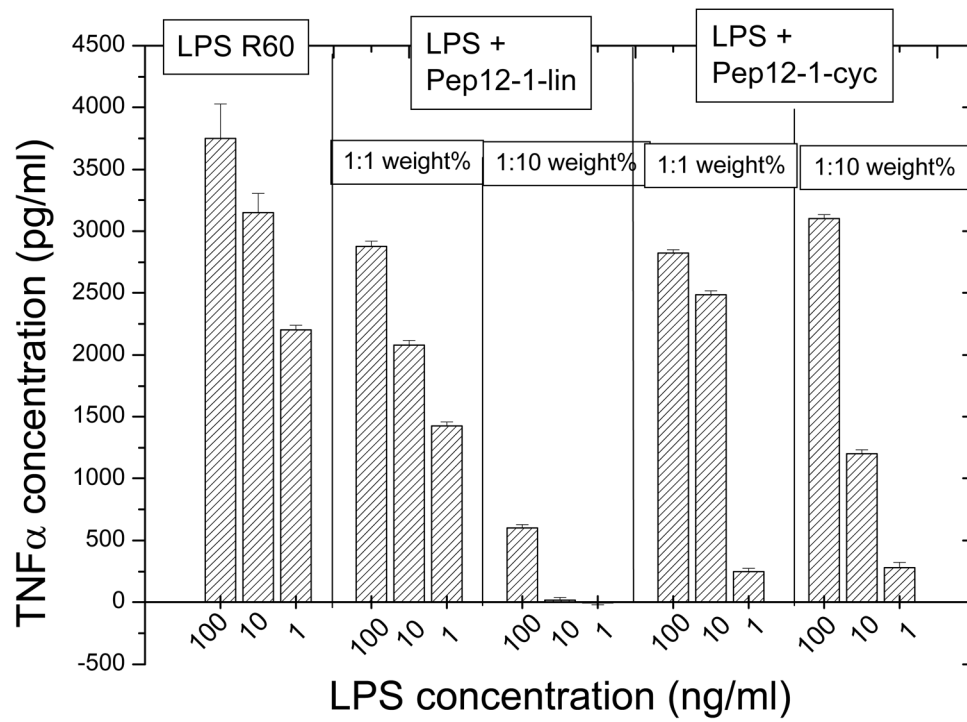


Figure 5. Inhibition of the LPS-induced production of tumor-necrosis-factor α in human mononuclear cells by peptides Pep12.1 in linear and cycled form at different [LPS]:[Pep] weight ratios.

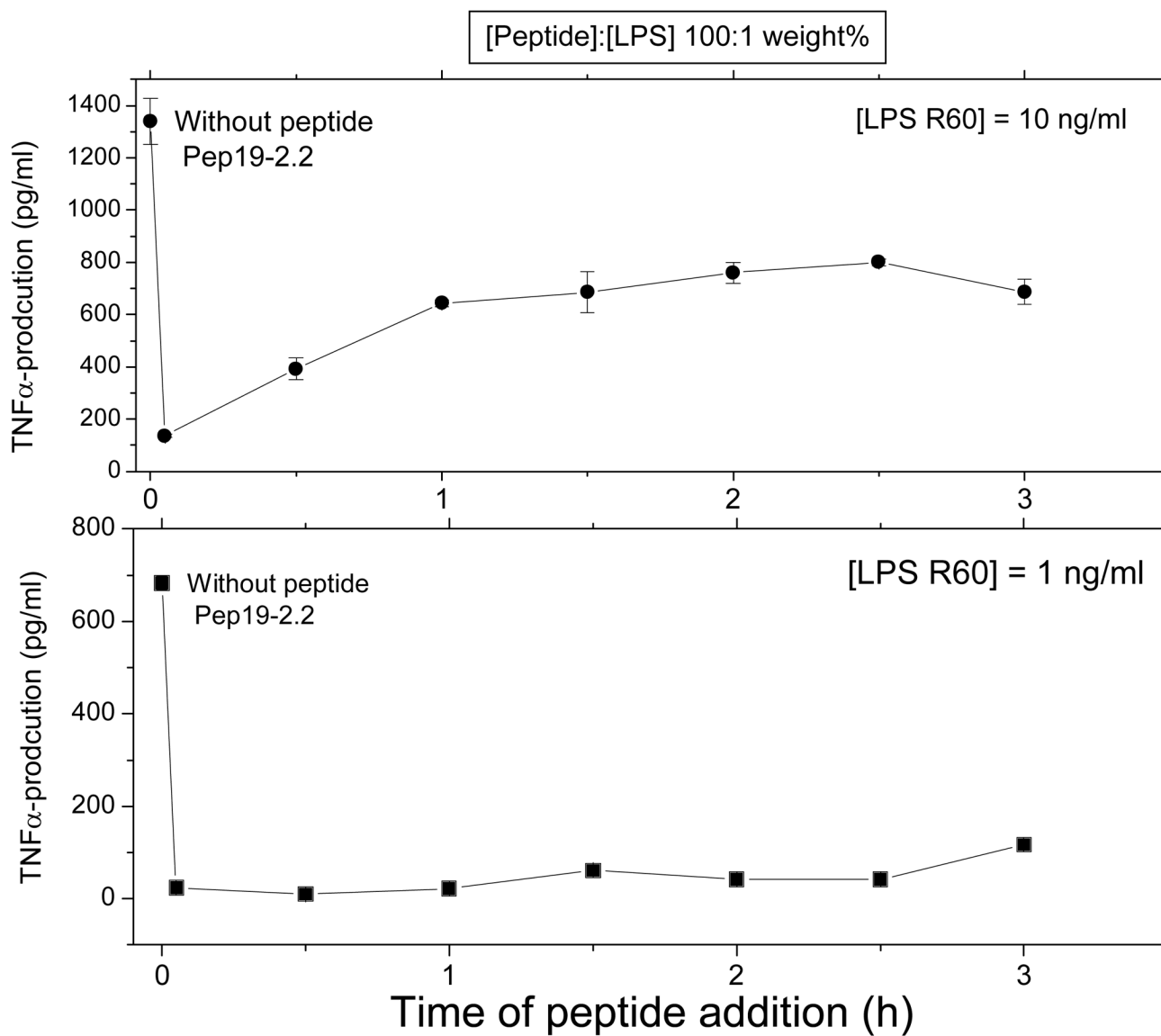
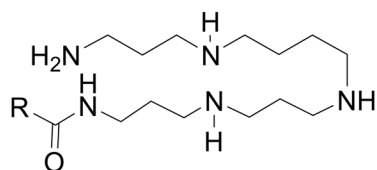
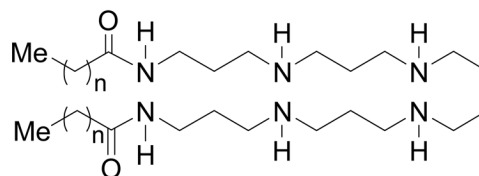


Figure 6. Inhibition of the LPS-induced production of tumor-necrosis-factor α in human mononuclear cells by peptide Pep19-2.2, at t=0 and added time-delayed up to 3 h later (entire stimulation time 4 hours). [LPS]:[Pep19-2.2] 1:100 weight%.

Monoacyl-homospermines.

4a, R = CH₃; **4b**, R = C₈H₁₇;
4c, R = C₉H₁₉; **4d**, R = C₁₂H₂₅;
4e, R = C₁₄H₂₉; **4f**, R = C₁₆H₃₃;
4g, R = C₁₈H₃₇

Bisacyl-homospermines.

8a, n = 7; **8b**, n = 8; **8c**, n = 10;
8d, n = 13; **8e**, n = 15; **8f**, n = 17

Figure 7.

Structures of the mono-acyl (**4** series) and *bis*-acyl (**8** series) lipopolyamines (top).
 Correlation of MIC against *S. aureus* and *E. coli* with length of the acyl group.

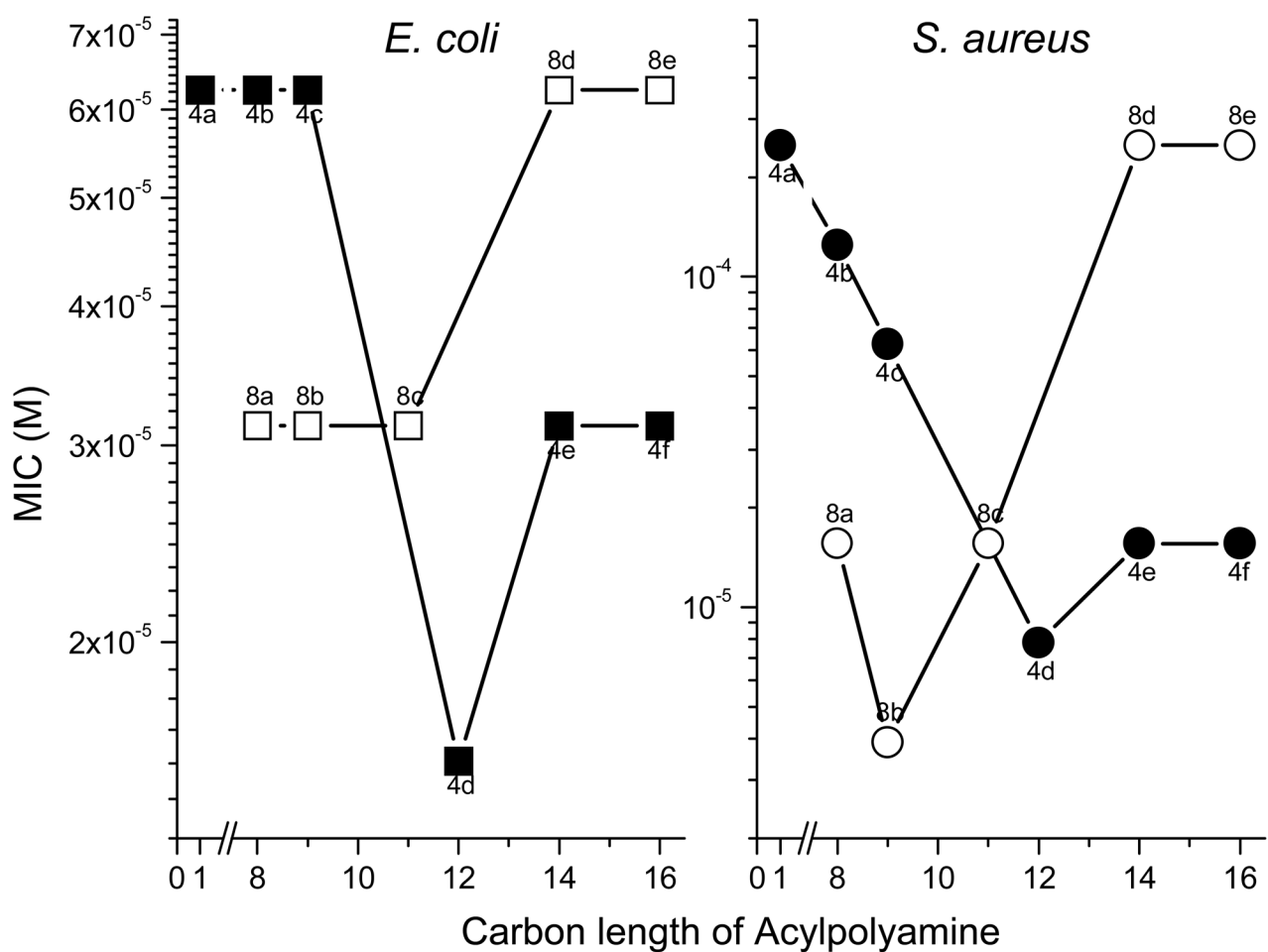


Figure 8.
Antimicrobial activity of various lipopolyamines against *E. coli* and *S. aureus*.

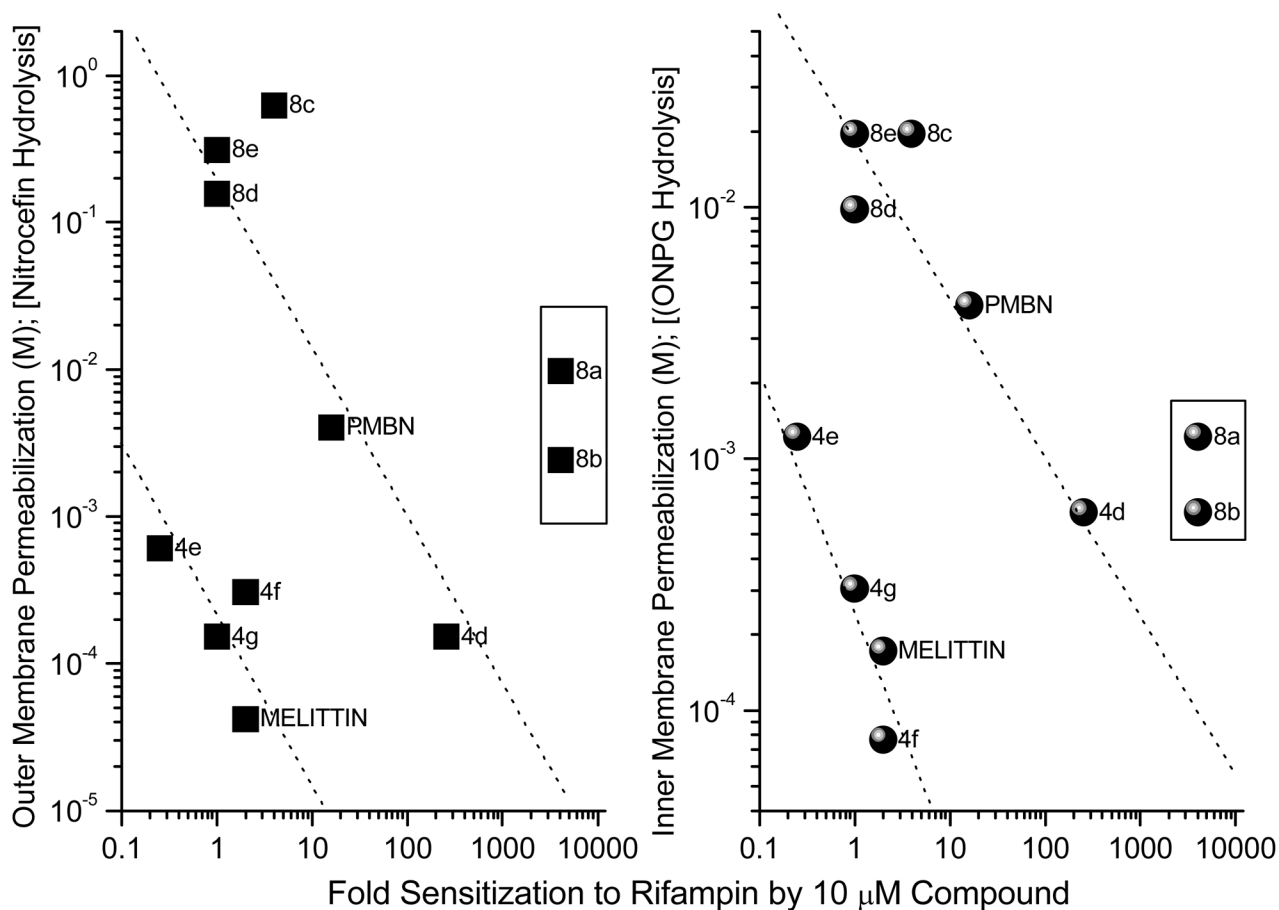


Figure 9.

Correlation of outer- (OM) and inner-membrane (IM) permeabilizing activity. OM permeabilizing activity was determined using *E. coli* ML-35p (parent ATCC 43827 transformed with pBR322 vector encoding periplasmic β -lactamase); the leakage of periplasmic β -lactamase activity was quantified using nitrocefin as a chromogenic substrate. IM permeabilizing activity was determined using *E. coli* ML-35 using *o*-nitrophenyl- α -D-galactopyranoside as the substrate to determine the α -galactosidase activity. Polymyxin B, Polymyxin B nonapeptide (PMBN) and melittin were used as reference compounds.

Antibacterial and antiendotoxic activities of the peptides and their N-acyl derived Lipopeptides

Table 1

Compound	Sequence ¹	MIC (µg/mL) of compound on the indicated bacterial strain												
		Ec	Ec-2	Pa	Pa-2	Ab	Sm	Bb	Ba	Sa				
P2-15	F W R I R I R R -NH ₂	8	8	32	16	128	64	2	64	32				
LP3-55	octanoil-P2-15	32	16	64	64	64	256	8	8	16				
LP3-56	2-ethylhexanoil-P2-15	16	8	32	64	64	128	8	8	16				
LP3-57	2,2-dimethylbutanoil-P2-15	8	8	32	8	64	128	4	8	8				
LP3-58	6-metiloctanoil-P2-15	32	16	64	256	64	256	16	8	64				
LP3-79	diclohexilacetil-P2-15	32	64	256	64	256	>256	8	8	32				
P2-27	F W R F W R R -NH ₂	16	64	64	32	128	256	4	16	16				
LP3-60	octanoil-P2-27	64	128	128	128	256	256	8	8	128				
LP3-63	6-metiloctanoil-P2-27	64	64	256	128	256	128	8	16	16				
P2-29	F W R I W R R -NH ₂	16	16	32	8	32	128	4	16	8				
P2-33	P F W R I R I R R -NH ₂	16	64	64	32	128	256	4	16	16				
LP3-53	octanoil-P2-33	64	128	128	128	256	256	8	8	128				

¹ Parent peptides and their N-acyl derivatives were synthesized with an amidated C-terminus

² Minimum inhibitory concentration of the compound determined by a microbroth-based assay in Mueller-Hinton medium. Abbreviations: Ec, *Escherichia coli* ATCC 25922; Ec-2; *Escherichia coli* DC2; Pa, *Pseudomonas aeruginosa* 4158-02 CUN; Pa-2, *Pseudomonas aeruginosa* ATCC 27853; Ab, *Acinetobacter baumannii* ATCC 19606; Sm, *Stenotrophomonas maltophilia* 3998-00 CUN; Bb, *Bordetella bronchiseptica* 11844-99 CUN; Ba, *Bruceella abortus* 9,49 perA; Sa, *Staphylococcus aureus* ATCC 25923; MRSA, *Staphylococcus aureus* ATCC 43300 (MRSA)

³ novobiocin enhancing activity of peptides on *Pseudomonas aeruginosa* Ps4 expressed as Fractional Inhibitory Concentration (FIC) index. FIC index was calculated according to the following formula: [(A)/MIC_A] + [(P)/MIC_P] = FICA + FIC_P = FIC index, where MIC_A and MIC_P are the MICs of the antibiotic and peptide determined separately, and (A) and (P) are the MICs of the antibiotic and peptide when determined in combination

⁴ number on the left: percentage of killing by the compound (MTT test; 4 h of treatment) when the peptide was added at 10 times its MIC on a biofilm of *Pseudomonas aeruginosa* PAO1; number on the right: percentage of biofilm removal determined by crystal violet staining under the same conditions of the killing assay. Values of standard deviation were always below 11%. n.d. not determined

⁵ survival of CD-1 mice after 7 days of being inoculated intraperitoneally (i.p) with 0.3 µg of lipopolysaccharide from *Pseudomonas aeruginosa* PAO1 followed 1 min later by the compound (150 µg, i.p.); galactosamine (18 mg) was co-inoculated with the lipopolysaccharide to sensitize the animals to endotoxin. -, +, ++, +++ denotes the level of protection (null, low, intermediate, high, respectively) afforded by the compound with respect to a control group of animals receiving no treatment

Table 2

Sequences of the Pep19 series

• Pep9	FRRLKWKFW
• Pep12-2	FRRLKWKKFWFW
• Pep17-1	KKFRRLKWKYKGFWW
Pep17-2	KKYRRFRWFKGKFWFW
Pep17-3	RRYKKFKWRYRGRFWFW
• Pep19-2:	GCKKYRRFRWFKGKFWFCG
• Pep19-2.2	GCKKYRRFRWFKGKFWFW
• Pep19-2.5	GCKKYRRFRWFKGKFWFWG
• Pep19-5:	GCKKYRRFRWFKRGRFWFWG
• Pep19-6:	GCKKFRFRLKCKQLWLWCG
Pep19-7:	GCKYRRFWFKGKFWFWG
• Pep19-12:	GCRRFKFKKWRGRFWFCG
• Pep19-12.1	GCKKFRFRRWKYKGFWWFCG
• Pep19-12.1cyclic	GCKKFRFRRWKYKGFWWFCG

Table 3

Survival of mice

Peptide	Dead mice/Number of mice after 48 h
LPS alone	10/10
LPS + Polymyxin B	0/10
LPS + Pep17-1	7/10
LPS + Pep17-2	7/10
LPS + Pep19-2	0/10