Identification from a Positional Scanning Peptoid Library of in Vivo Active Compounds That Neutralize Bacterial Endotoxins

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Two peptoids that neutralize the Gram-negative lipopolysaccharide (LPS) were identified from the screening of a positional scanning library. The evaluation of the in vivo activity of these compounds in an endoxemia murine model is also reported. These peptoids did not neutralize lipid A, i.e., the hydrophobic toxic component of LPS. This fact suggests that they do not have access to the micellar core and that they should bind to the hydrophilic carbohydrate portion of LPS.

Introduction

Gram-negative bacterial lipopolysaccharide (LPS) or endotoxin is a potent initiator of the innate inflammatory response.[1] LPS induces the production of proinflammatory cytokines and chemokines, and it is recognized as a key molecule in the pathogenesis of endotoxin shock,[2] a leading cause of mortality in septic patients. No specific drugs to decrease LPS levels are as yet available; thus, the identification of new lead compounds that may be developed in efficacious and safe LPS-neutralizing molecules is a matter of intensive research. The mechanism underlying LPS-induced activation of inflammatory cells requires the intervention of at least four proteins to trigger the sensitive cellular response. The LPS binding protein (LBP) present in the blood stream binds to LPS micelles. It has been postulated that LBP extracts the LPS monomers and that these monomers are transferred to the soluble CD14, also present in the blood.[3] The complex is presented to the membrane-bound form of CD14, and the newly formed complexes are thought to initiate intracellular signaling reactions by binding to Toll-like receptors (TLRs) on macrophages and other cells.[4] It has been reported that different families of cationic antimicrobial peptides that block LPS-induced macrophage activation can bind to LPS and inhibit the binding of LPS to LBP, thus preventing the LBP-mediated transfer of LPS to CD14.[5−8] Furthermore, it has also been hypothesized that the complex LBP monomeric LPS could serve as an alternative detoxification method involving the participation of additional lipid binding proteins.[9] Thus, the design of small organic molecules that could bind to LPS and neutralize its toxic action may lead to new drugs useful for the treatment of endotoxin shock in the context of a multitherapy strategy.

Oligomers of *N*-alkylglycines, also known as peptoids, constitute a family of non-natural molecules attractive for the drug discovery process because of their broad variety of biological activities and the proteolytic stability that they exhibit.[10] As part of our program to generate a diverse collection of combinatorial libraries addressed to protein/protein and protein/lipid interactions, we early designed a library of peptoids in a positional scanning format.[11,12] The availability of a peptoid library in such a format allowed the use of the same library in different biological targets for the identification of lead compounds.[13−15] We have improved the synthetic methodology to generate a less chemical-redundant and more biological-sense directed version of the original library. The details of the design principles and synthesis of this new library will be reported elsewhere.

We present herein the results of the screening of this improved peptoid library containing 5120 peptoids for the identification of molecules that exert protective action against endotoxin shock by neutralizing the LPS toxin. The trimeric library was organized into 52 controlled mixtures. Mixtures 1−20 (O_1XX) contained as defined position 1 of 20 commercially available primary amines, most of them selected for the original peptoid library, while at the "X" positions (mixture positions) a set of 16 primary amines was used. Mixtures 21−36 (XO_2X) and 37−52 (XXO_3) contained at the defined position only the 16 amines set. The LPS-neutralizing activity of each peptoid mixture making up the three sublibraries was estimated by the chromogenic *Limulus* amebocyte lysate assay,[16] which allows the detection of free LPS. The results of the screening of the peptoid library are shown in Figure 1A from which we selected the most active mixtures to define IC_{50} values by a 2-fold serial dilution assay. Thus, at the "O_1" position, the selected amines were phenethylamine (from mixture 7, IC_{50} = 350 μM), tetrahydrofurfurylamine (from mixture 8, IC_{50} = 200 μM), and 3,3-diphenylpropylamine (from mixture 14, IC_{50} = 90 μM). At the "O_2" position they were 4-methoxyphenethyl-

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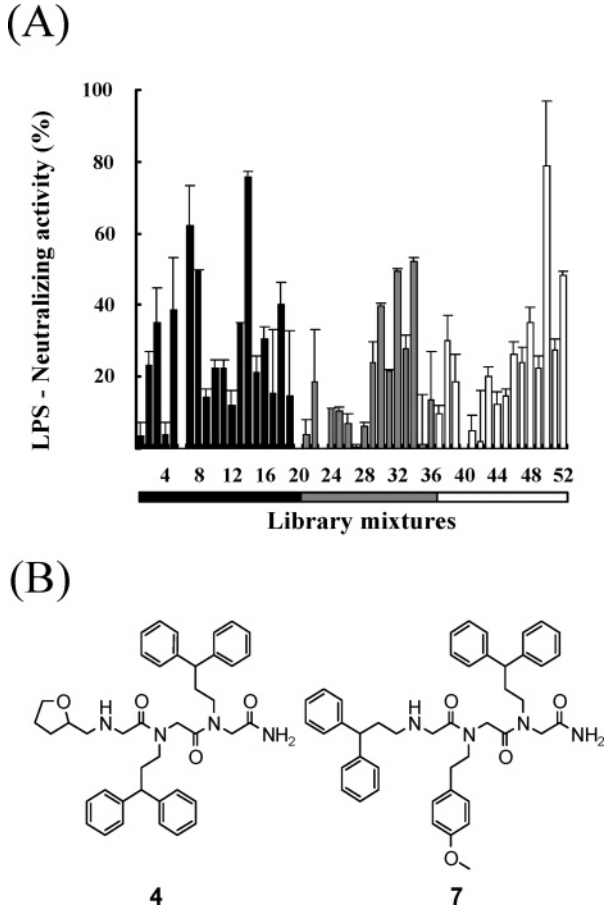

Figure 1. (A) Screening of the library for the identification of peptoids that neutralize LPS: neutralizing activity profile of the library mixtures. Each bar in the panel represents the neutralizing value for each peptoid mixture, with the *x*-axis representing the defined amine ("O") position. Black bars are the values for mixtures with the first position defined (OXX), and gray and white bars are for XOX and XXO, respectively. (B) Structures of peptoids **4** and **7**.

amine (from mixture 30, $IC_{50} = 160$ μM), 2,4-dichlorophenethylamine (from mixture 32, $IC_{50} = 240$ μM), and 3,3-diphenylpropylamine (from mixture 34, $IC_{50} = 80$ μM). Finally, at the "O_3" position, 2,4-dichlorophenethylamine (from mixture 48, $IC_{50} = 150$ μM), 3,3-diphenylpropylamine (from mixture 50, $IC_{50} = 150$ μM), and 4-fluorophenethylamine (from mixture 52, $IC_{50} > 350$ μM) were selected. Thus, we finally opted for tetrahydrofurfurylamine and 3,3-diphenylpropylamine at "O_1", 4-methoxyphenethylamine and 3,3-diphenylpropylamine at "O_2", and 2,4-dichlorophenethylamine and 3,3-diphenylpropylamine at "O_3". This deconvolution led to the synthesis of eight discrete compounds (peptoids **1**−**8**, Table S1 in Supporting Information). The individual peptoids were initially tested for LPS-neutralizing activity, and their activity was compared with the high-affinity LPS peptide binders polymyxin B (PMB) [17] and LALF-14 [18] (Table 1). The results showed that these newly identified peptoids are potent in vitro LPS-neutralizing compounds. The two most active peptoids, namely, **4** and **7** (Figure 1B), were selected for further assays.

The basic LPS molecular structure consists of two distinct regions: a hydrophilic carbohydrate portion and outer core region;and the hydrophobic toxic lipid A component. This latter component is highly conserved among Gram-negative bacteria, and it has been reported that it contributes to the toxicity of LPS.[19] In contrast with the mode of action of previously reported LPS-neutralizing synthetic peptides,[16,20−23] **4** and **7** did not neutralize *E. coli* 1,4′-diphosphoryl lipid A (DPLA) in the in vitro assay (Table 1), suggesting that the peptoids do not have access to the micellar core and should bind to the hydrophilic carbohydrate portion of LPS.

Table 1. Biological Activity of Defined Peptoids Rescued from the Library

peptoid	LPS-neutralizing activity (IC_{50}, μM)[a]	DPLA-neutralizing activity (IC_{50}, μM)[a]	quantitation of TNF-α release in mice challenged with LPS or premixed LPS/peptoid[b]
1	>250	ND[c]	ND[c]
2	>250	ND[c]	ND[c]
3	70	150	ND[c]
4	40	250	71 ± 22
5	125	>250	ND[c]
6	>250	ND[c]	ND[c]
7	50	>250	10 ± 6
8	NDS[d]	NDS[d]	NDS[d]
PMB[e]	$<10^{-4}$	ND[c]	5 ± 3
LALF-14[f]	64	40	67 ± 42

[a] IC_{50} = concentration necessary to in vitro neutralize 50% of LPS or of DPLA as determined by a serial dilution assay. [b] Data are in % of TNF-α measured in the presence of peptoid or PMB with respect to that measured when animals were treated only with LPS (100%). Mice were ip injected with 10 μg of LPS, alone or premixed with 20 μg of each peptoid (in PBS containing 40% of DMSO) or 100 μg of PMB or LALF-14. After 1 h, mice were bled and collected sera were assayed for TNF-α content by ELISA. Data represent the mean ± SD of seven mice per group. [c] ND, not determined. [d] NDS, not determined because of solubility problems. [e] Cyclized isooctanoyl BTBB(BfLBBT). Single letter amino acid code for amino acids: B, diaminobutyrate; f, D-phenylalanine. Sequence in parentheses is cyclized. [f] G(CKPTFRRLKWKYKC)G. Sequence in parentheses is cyclized.

We further examined the effects of peptoid **4** (peptoid **7** was extremely insoluble in pure buffered solutions) on the micellar organization of lysophosphatidylcholine (LPC, a model lysophospholipid with a well-characterized critical micellar concentration, cmc),[24] LPS, and DPLA. The fluorescent probe 1,3-diphenyl-1,3,5-hexatriene (DPH) has been used as a fluorescent reporter molecule in studies of the effect of extrinsic molecules on the hydrophobic core of lipid vesicles[25] and on the cmc of surfactants.[26] The fluorescence emission spectrum of DPH depends on the polarity of the environment. Upon titration with LPC of a buffered solution of DPH (empty squares in Figure 2A), we obtained a fluorescence titration curve with a characteristic inflection point that marks for the cmc value of LPC. Such a value is in close agreement with previously reported data.[24] Peptoid **4** does not modify the cmc value of LPC as deduced from the analysis of the fluorescence titration curve obtained when a solution of DPH containing 40 μM peptoid **4** was titrated with LPC (filled squares in Figure 2A). Similar results were obtained when the integrity of the micellar organization of LPS and DPLA was analyzed in the absence (empty triangles and circles, in parts B and C of Figure 2, respectively) and in the presence of peptoid **4** (filled triangles and circles in parts B and C of Figure 2, respectively). These results are a further indication for the preferred surface localization of peptoid **4** (and probably peptoid **7**) in LPS micelles and contrast with those previously obtained for synthetic LPS-neutralizing peptides that induced major

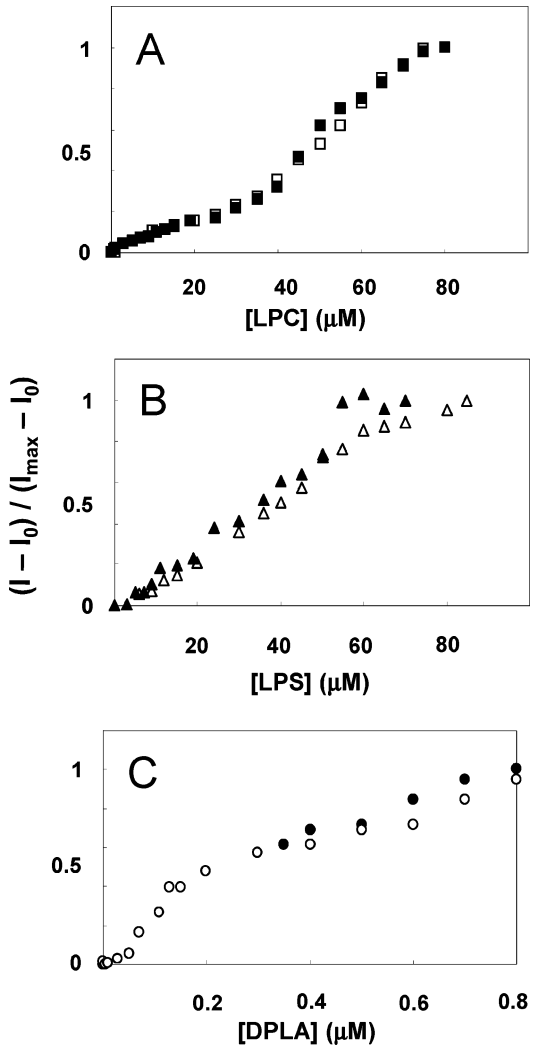

Figure 2. Effect of the LPS-neutralizing peptoid **4** on the cmc of different lipids illustrated by the fluorescence emission intensity of the probe DPH (measurements were carried out at 25 °C in 10 mM phosphate buffer, 137 mM NaCl, 3 mM KCl, pH 7.3) at 430 nm upon excitation at 380 nm in the absence (empty symbols) and in the presence of 40 μM peptoid **4** (filled symbols) at different lipid concentrations: (A) LPC; (B) LPS; (C) DPLA. The relative variation of the fluorescence intensity is expressed as the ratio $(I - I_0)/(I_{max} - I_0)$ where I_0 is the intensity at 430 nm in the absence of lipid, I is the fluorescence intensity at each lipid concentration, and I_{max} is the maximum fluorescence intensity obtained at saturating lipid concentrations.

perturbation of the micellar organization of DPLA and LPS.[16] In addition, peptoid **4** does not have antimicrobial activity as determined in standard antimicrobial assays (results not shown).

Several studies have consistently reported elevated levels of TNF-α in a large number of septic shock patients experiencing early death or suffering of prolonged fatal multiple organ failure.[27] By using a murine endoxemia model[18] where animals were injected with premixed LPS/peptoid samples (Table 1), we obtained initial evidence of potential in vivo activity of these compounds, in particular peptoid **7**, that under these conditions significantly decreased the serum levels of TNF-α. Nevertheless, peptoids were subjected to a more demanding in vivo assay. LPS was injected at the left side of the peritoneal cavity, while peptoids were injected at the right side. Peptoid **7** was able to inhibit TNF-α induction in a dose-response manner (Figure 3).

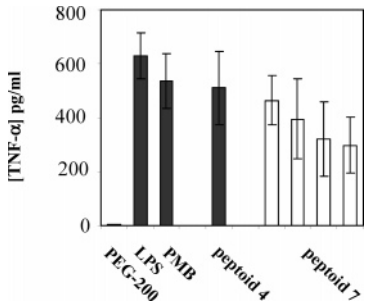

Figure 3. Inhibition of LPS-induced TNF-α release in mice by peptoids **4** and **7**. Mice were injected in the left side of the peritoneal cavity with 500 μg of LPS in PBS. After 20 s peptoids (pepotid **4**, 20 μg; peptoid **7** (white bars, 2, 6, 20, and 60 μg from left to right, respectively)) solubilized in PEG-200 or PMB (100 μg) were injected in the right side of the peritoneal cavity. After 1 h, mice were bled and collected sera were assayed for TNF-α content by ELISA. Data represent the mean ± SD of seven mice per group. A control group was injected with vehicle (PEG-200). The one-way ANOVA ($p < 0.05$) test with Scheffe post hoc correction was used for statistics analysis, and results from peptoids treatment were considered significantly different from results from the control.

In conclusion, from the screening of a positional scanning library, we have identified active peptoids that neutralize LPS. To the best of our knowledge, this peptoid family could represent the first example of how small molecules could be addressed to interfere at lipid/protein interactions of biological relevance. In light of our results, we propose that peptoids **4** and **7**, in their positively charged forms at physiological pH (with pK_a and M log P values of 7.2, 3.5, and 8.0, 4.6, respectively), bind to the external negatively charged hydrophilic carbohydrate portion of LPS that would induce a biological active conformation on the peptoids. Our results also shown that peptoids **4** and **7** do not bind to the lipid A component of LPS and do not induce micellar core perturbation effects. This fact could be related to its low toxicity toward Gram-negative bacteria, even though these peptoids elicit in vivo activity in an endoxemia murine model and decrease LPS-dependent TNF-α production. Although there are questions that still need to be addressed, LPS-neutralizing peptoids could become one of the new classes of compounds that can be used to increase the susceptibility of Gram-negative bacteria to hydrophobic antibiotics by synergistic processes[28–30] and for the treatment of endotoxin shock in a multitherapy regimen.

Experimental Section

Abbreviations. BPI, bactericidal/permeability-increasing protein; cmc, critical micellar concentration; DPLA, 1,4′-diphosphoryl lipid A; LALF, *Limulus* anti-LPS factor; LAL, *Limulus* amebocyte lysate; LPC, lysophosphatidylcholine; LPS, lipopolysaccharide; LBP, lipopolysaccharide binding protein; PBS, phosphate-buffered saline; PMB, polymyxin B.

Chemistry. The details on the synthesis of the peptoid library will be reported elsewhere. The synthesis of individual peptoids is described in the Supporting Information.

LPS Neutralizing Assays. The in vitro neutralizing activity of the library and of the defined peptoids was measured as previously described.[16] Experiments performed for the analysis of the inhibition of LPS-induced TNF-α release in mice are described in the Supporting Information.

Fluorescence Spectroscopy Studies. The determination of the cmc for the three different lipids (LPC, DPLA, and LPS) in the presence and in the absence of peptoid **4** was obtained by analyzing the fluorescence emission spectra (Perkin-Elmer LS-50 spectrofluorimeter) of DPH.

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Supporting Information Available: Table S1, experimental procedures, and NMR, RP-HPLC, and HRMS data for **4** and **7**. This material is available free of charge via the Internet at http://pubs.acs.org.

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