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Functionalized dendrimers as endotoxin sponges

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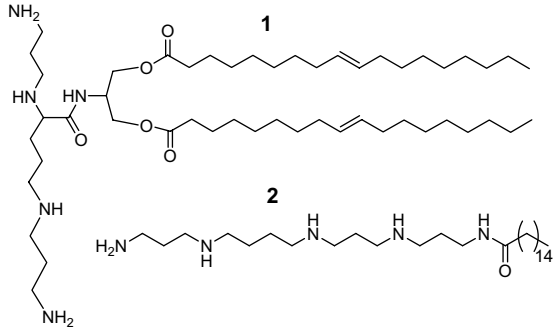
Abstract—Lipopolysaccharides (LPS), otherwise termed 'endotoxins', are outer-membrane constituents of Gram-negative bacteria, and play a key role in the pathogenesis of 'Septic Shock', a major cause of mortality in the critically ill patient. We had previously defined the pharmacophore necessary for small molecules to specifically bind and neutralize LPS and, using animal models of sepsis, have shown that the sequestration of circulatory LPS by small molecules is a therapeutically viable strategy. Polyamidoamine dendrimers, with the surface amines substoichiometrically derivatized with alkyl groups bind LPS with high affinity, neutralize LPS-induced inflammatory responses in vitro, and afford protection in a murine model of endotoxic shock. Dendrimers represent a new class of potentially useful compounds for the therapy of Gram-negative sepsis.

Endotoxins, or lipopolysaccharides (LPS), the predominant structural component of the outer membrane of Gram-negative bacteria,[1] play a pivotal role in septic shock, a frequently fatal syndrome of systemic inflammation, which accounts for more than 200,000 fatalities in the US annually.[2] Despite tremendous strides in antimicrobial chemotherapy, the incidence of sepsis has risen almost three-fold from 1979 to 2000[3] and sepsis-associated mortality has essentially remained unchanged at about 45%, emphasizing an urgent, unmet need to develop therapeutic options specifically targeting the pathophysiology of sepsis. The toxicity of LPS resides in its structurally highly conserved glycolipid component called Lipid A, a glycolipid composed of a hydrophilic, bis-phosphorylated diglucosamine backbone, and a hydrophobic domain of 6 (*E. coli*) or 7 (*Salmonella*) acyl chains[4–6] (Fig. 1). The anionic and amphiphilic nature of lipid A enables it to bind to a variety of cationic amphipaths.[7–14] We have shown that the pharmacophore necessary for optimal recognition and neutralization of lipid A by small molecules requires two protonatable positive charges so disposed that the distance between them are equivalent to the distance between the two anionic phosphates on lipid A (~14 Å), enabling ionic H-bonds between the phosphates on the lipid A backbone and the positive charges on the compound. In addition, appropriately positioned pendant hydrophobic functionalities are necessary to stabilize the resultant complexes via hydrophobic interactions with the polyacyl domain of lipid A; these lipophilic groups are necessary to convert lipid A binders to true endotoxin neutralizers.[11,10] Simple lipopolyamines such as DOSPER **1** (1,3-dioleoyloxy-2-(6-carboxyspermyl)-propylamide),[12] and monoacyl homologated spermine **2**,[8] embodying such structural features, effectively neutralize LPS in vitro as well as in animal models of endotoxin-induced shock.



Using a high-throughput screening strategy,[15] we have recently identified branched dendrimeric architectures[16] as potential lead scaffolds for strong lipid A binding.[17]

Keywords: Lipopolysaccharide; Endotoxin; Sepsis; Dendrimers.
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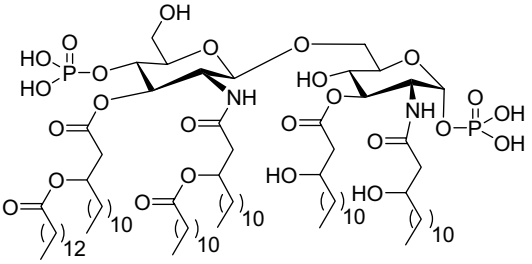
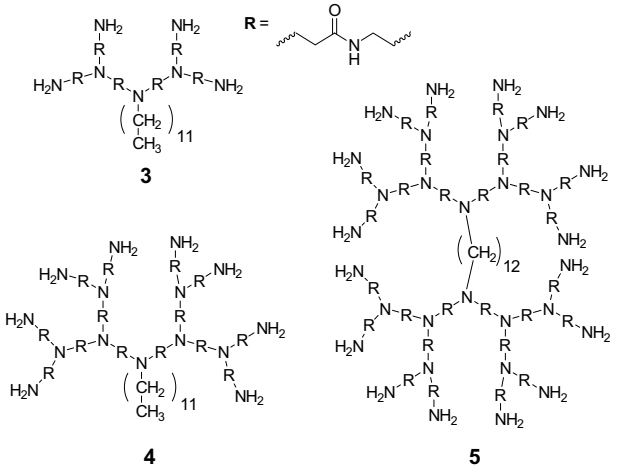


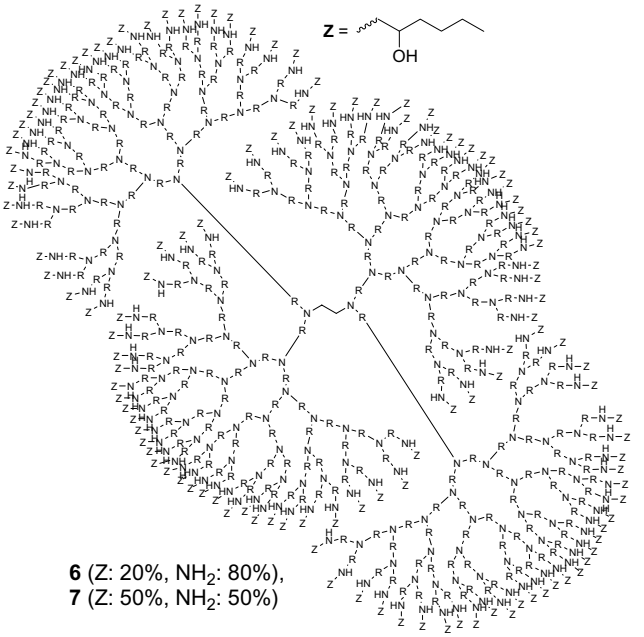
Figure 1. Structure of Lipid A, the toxic principle of lipopolysaccharide.

We reasoned that the polycationic amine-terminated dendrimers which are suitably functionalized with hydrophobic groups would present multiple lipid A neutralizing sites, and may behave as synthetic surrogates for basic, hydrophobic proteins such as the *Limulus polyphemus*-derived endotoxin neutralizing protein[18–20] or the neutrophil granule-derived bactericidal/permeability increasing protein (BPI)[21–24], both of which are in clinical trials.

A variety of amine-terminated poly(amidoamine) (PAMAM) dendrimers[25] of varying generations and core functionalities, and with surface groups modified with varying degrees of substitution with diverse hydrophobic groups were procured from Dendritech, Inc. (Midland, MI), and were used as supplied. These include compounds with cores such as 1-aminododecane (**3**, **4**), 1,12-diaminododecane (**5**), or ethylenediamine (**6**, **7**), and are thus, respectively, monodendrons (**3**, **4**), didendron (**5**), or true, multi-branched dendritic structures (**6**, **7**).[25,26] The terminal amino groups ($n = 128$ in Generation 5 dendrimers such as in **6** and **7**) were alkylated by Dendritech to 25%, 50%, and 75% with 1,2-epoxyalkanes (resulting, for instance, in *N*-substituted 2-hydroxyhexyl groups in **6** and **7**).[27] The degree of alkylation was verified by assaying for primary amino groups using fluorescamine.[28] For the sake of brevity, results obtained with a relevant subset of the dendrimers are presented.



3 **4** **5**



6 (Z: 20%, NH_2: 80%),
7 (Z: 50%, NH_2: 50%)

Quantitative determination of binding affinity of the dendrimers to LPS was performed using a recently described[15] high-throughput fluorescence displacement method, using BODIPY cadaverine (BC) (5-(((4-(4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-yl) phenoxy)acetyl)amino)pentylamine, hydrochloride) as the displacement probe. In these experiments, polymyxin B (PMB), a decapeptide antibiotic known to bind specifically to the lipid A moiety of LPS and neutralize the toxicity of LPS[7] was used as a reference compound. Concentrations of the dendrimers causing 50% effective displacement of BC (ED_{50} values), depicted in Figure 2 indicated that **7** was the highest-affinity binder with an ED_{50} of 91 nM. Compound **6** also displayed a higher affinity (ED_{50}: 0.28 μM) than PMB (0.31 μM). Non-derivatized PAMAM dendrimers bound LPS with considerable lower affinities (data not shown) suggesting that the surface hydrophobic groups contributed significantly to binding affinity, consistent with our previous observations[29] with bis-cationic compounds[10,11] and polyamines.[8,9,12,13] It is of interest that 50% derivatization of the Generation 5 dendrimer with 2-hydroxyhexyl groups (**7**) is optimal in terms of binding affinity; alkylation of a higher percentage of surface amino groups, or derivatization with C_8, C_{10}, or C_{12} groups resulted in compromised aqueous solubility, which dramatically lowers the binding affinity (data not shown).

The biological activity of the dendrimers was first examined in an in vitro assay of nitric oxide (NO) release in LPS-stimulated murine macrophage J774A.1 cells.[12,8] Compounds that bind and sequester LPS show a concentration-dependent inhibition of LPS-induced NO production (measured as nitrite[30] using the Greiss reagent[31]). As shown in Figure 3, **7** is the most potent of the dendrimers tested, with a 50% inhibition constant

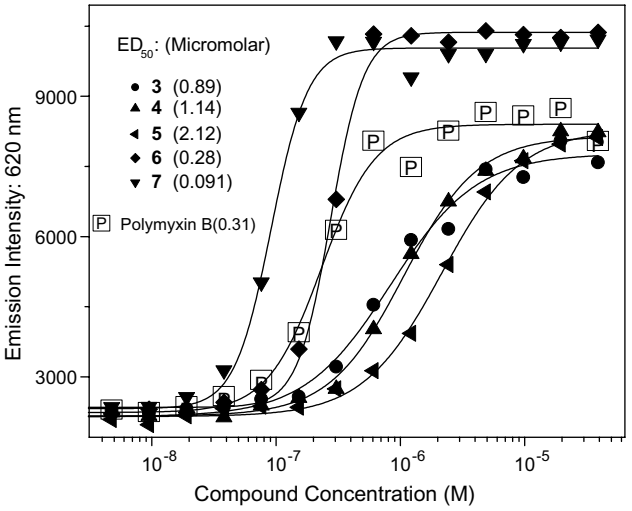


Figure 2. Affinity of binding of dendrimers to lipopolysaccharide.

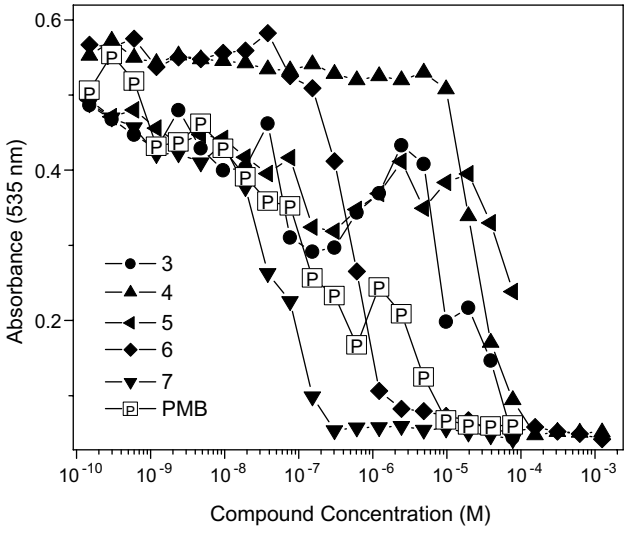


Figure 3. Inhibition by dendrimers of LPS-induced nitric oxide production in mouse macrophage cell-line, J774A.1.

(IC_{50}) of 50 nM; the IC_{50} of the reference compound, PMB, was found to be 210 nM. Consistent with the fluorescence displacement assay results, the IC_{50} values of the native, underivatized PAMAM dendrimers were in the 5–10 μM (data not shown), underlining the obligatory requirement of both cationic and hydrophobic groups for optimal neutralization of endotoxic activity.[10,12,11]

Given the high binding affinity and neutralization potency of **7**, and the fact that dendrimers have been successfully used as gene-transfer agents in mammalian cells,[32,33] suggesting that toxicity may not be a limiting factor,[34] we elected to evaluate the protective effects of **7** in greater detail using a murine model of LPS-induced septic shock.[12] A supralethal dose (twice the dose causing 100% lethality) of 200 ng/mouse was administered intraperitoneally to groups of 5 CF-1 mice sensitized with D-galactosamine, along with concurrent, separate subcutaneous injections of graded doses of **7**, and lethality was observed at 24 h. As shown in Table 1, a dose-dependent protection is observed, with full protection being evident at 200 μg per mouse.

Table 1. Dose-dependent protection by **7** against LPS-mediated lethality in D-galactosamine-primed mouse model

Dose of **7** in μg (subcutaneous)	No. of live/total mice at 24 h[a]
20	0/5
50	0/5
100	3/5
200	5/5*
500	5/5*

* Statistically significant by Fisher one-tailed exact probability test.
[a] The LD_{100} dose was determined to be 100 ng LPS per mouse. A supralethal dose of 200 ng/mouse was used.

In our earlier studies with the lipopolyamine **1**, we had obtained similar dose-dependent protection, but the duration of protection was very short, presumably due to the lability of the ester linkages of the lipophilic chains.[12] We surmised that the temporal window of protection with **7** may be greater, given the stability of the dendrimeric scaffold as well as the C_6 surface alkyl groups. We therefore conducted a time-course experiment in which **7** was administered at various times with respect to LPS challenge.

As shown in Table 2, statistically significant protection was observed at 6 h prior to LPS challenge. In longer time-course experiments, partial protection was observed up to 36 h preceding endotoxin administration (data not shown), indicating highly prolonged pharmacokinetics.

We have shown that dendrimers with polyamino surfaces that are partially modified by hydrophobic groups effectively bind and sequester bacterial lipopolysaccharides in vitro as well as in in vivo models of septic shock. The high affinity and potency as well as the prolonged apparent half life of dendrimers in systemic circulation render them a novel class of lipopolysaccharide sequestrants of potential therapeutic value.

Table 2. Temporal window of protection by **7** against LPS-mediated lethality in D-galactosamine-primed mouse model

Time of administration of **7** w.r.t LPS challenge[a] (h)	No. of live/total mice at 24 h following LPS challenge[a]
−6	4/5*
−4	5/5*
−2	4/5*
0	4/5*
+2	1/5

* Statistically significant by Fisher one-tailed exact probability test.
[a] A supralethal LPS dose of 200 ng/mouse was administered intraperitoneally at time zero. Five hundred micrograms of **7** was injected subcutaneously at the times indicated.

Acknowledgements

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2005.01.026.

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