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Lysine–spermine conjugates: hydrophobic polyamine amides as potent lipopolysaccharide sequestrants

Mark R. Burns,[a] Stewart J. Wood,[b] Kelly A. Miller,[b] Thuan Nguyen,[b] Jens R. Cromer[b] and Sunil A. David[b,*]

[a]*MediQuest, Inc., 4101 Stone Way North, Suite 220, Seattle, WA 98103, USA*
[b]*Department of Medicinal Chemistry, University of Kansas, Life Sciences Research Laboratories, 1501 Wakarusa Drive, Lawrence, KS 66049, USA*

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Abstract—Lipopolysaccharides (LPS), otherwise termed 'endotoxins', are outer-membrane constituents of Gram-negative bacteria. Lipopolysaccharides play a key role in the pathogenesis of 'Septic Shock', a major cause of mortality in the critically ill patient. Therapeutic options aimed at limiting downstream systemic inflammatory processes by targeting lipopolysaccharide do not exist at the present time. We have 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. In this paper, the interactions of a focused library of lysine–spermine conjugates with lipopolysaccharide (LPS) have been characterized. Lysine–spermine conjugates with the ε-amino terminus of the lysinyl moiety derivatized with long-chain aliphatic hydrophobic substituents in acyl or alkyl linkage bind and neutralize bacterial lipopolysaccharides, and may be of use in the prevention or treatment of endotoxic shock states.

1. Introduction

Endotoxins, or lipopolysaccharides (LPS), the predominant structural component of the outer membrane of Gram-negative bacteria,[1,2] play a pivotal role in septic shock, a syndrome of systemic toxicity, which occurs frequently when the body's defense mechanisms are compromised or overwhelmed, or as a consequence of antibiotic chemotherapy of serious systemic infections (Gram-negative sepsis).[3–5] Referred to as 'blood poisoning' in lay terminology, Gram-negative sepsis is the thirteenth leading cause of overall mortality[6] and the number one cause of deaths in the intensive care unit,[7] accounting for more than 200,000 fatalities in the US annually.[8] Despite tremendous strides in antimicrobial chemotherapy, the incidence of sepsis has risen almost threefold from 1979 through 2000[9] and sepsis-associated mortality has essentially remained unchanged at about 45%, both calling to attention the fact that aggressive antimicrobial therapy alone is insufficient in preventing mortality in patients with serious illnesses, and emphasizing an urgent, unmet need to develop therapeutic options specifically targeting the pathophysiology of sepsis.

The presence of LPS causes a widespread activation of the innate immune response,[10,11] leading to the uncontrolled production of numerous inflammatory mediators, including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6), primarily by cells of the monocyte/macrophage lineage.[12,13] The unregulated overproduction of these mediators, as well as others, such as nitric oxide produced by the endothelial cell,[14,15] leads to a systemic inflammatory response characterized by fever, hypotension, coagulopathy, hemodynamic derangement, tissue hypoperfusion, and multiple organ failure,[16,17] culminating frequently in death.

The therapy of septic shock remains primarily supportive, and specific modalities aimed at limiting the underlying pathophysiology are, unfortunately, as yet unavailable. One possible approach to addressing

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* Corresponding author. Tel.: +1 785 330 4316; fax: +1 785 330 4332; e-mail: sdavid@ku.edu

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therapeutically the problem of Gram-negative sepsis has been to target LPS itself by the use of an agent that would bind to, and sequester it. It has been shown by total synthesis[18–21] that the toxicity of LPS resides in its structurally highly conserved glycolipid component called Lipid A.[22,23] Lipid A is composed of a hydrophilic, bis-phosphorylated diglucosamine backbone, and a hydrophobic domain of 6 (*E. coli*) or 7 (*Salmonella*) acyl chains in amide and ester linkages[24–26] (Fig. 1). The anionic and amphiphilic nature of lipid A (Fig. 1) enables it to bind to numerous substances that are positively charged and also possess amphipathic character. We have, over the past decade, characterized the interactions of lipid A with a number of classes of cationic amphipathic molecules including proteins,[27,28] peptides,[29–33] pharmaceutical compounds,[34,35] and other synthetic polycationic amphiphiles.[36–38] Importantly, from these and currently ongoing studies, we have determined the pharmacophore necessary for optimal recognition and neutralization of lipid A[35] 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 further enhance binding affinity and stabilize the resultant complexes via hydrophobic interactions with the polyacyl domain of lipid A (for a recent review, see Ref. 39). These structural requisites were first identified in certain members of a novel class of compounds, the lipopolyamines, which were originally developed, and are currently being used as DNA transfection (lipofection) reagents.[40–43] Compounds of the conjugated spermine class are of particular interest because they are active in vivo and afford protection in animal models of Gram-negative sepsis, are synthetically easily accessible, and, importantly, are nontoxic, on account of their degradation to physiological substituents (spermine and fatty acid).[37,44]

Ongoing research in our laboratory seeks to systematically identify structural variations in the polyamine backbone that would impart additional, enthalpically-driven H-bond/van der Waals interactions. The lysine–spermine derivatives described in this report exemplify a group of compounds that incorporate stereogenic H-bond donor/acceptor functionalities at one end of the polyamine scaffold. Besides confirming the obligatory requirement of a terminally-placed long-chain hydrophobic group for optimal endotoxin sequestration, we find significant differences in both the binding affinity and neutralization potency of L- and D-lysine conjugates, suggesting that an iterative substitution of the polyamine backbone with H-bond donor/acceptor functionalities with appropriate stereochemistry may yield highly potent, yet nontoxic endotoxin neutralizers.

2. Results and discussion

2.1. Chemistry

The method used for the synthesis of lysine–spermine conjugates enabled selective functionalization of the ε-nitrogen atom of lysine and chromatographic purification prior to exposure of the extremely polar amine groups. Specifically, blockage of the polar amino groups on the polyamine conjugates used Boc-carbamates, allowing normal-phase SiO_2 chromatography instead of the more time-consuming ion-exchange method previously reported.[45] Synthesis of these analogues, shown in Scheme 1, began by coupling the free base of sperm-

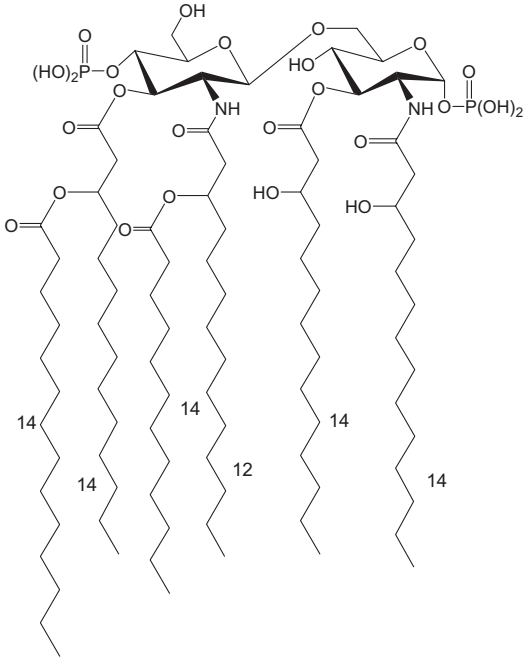
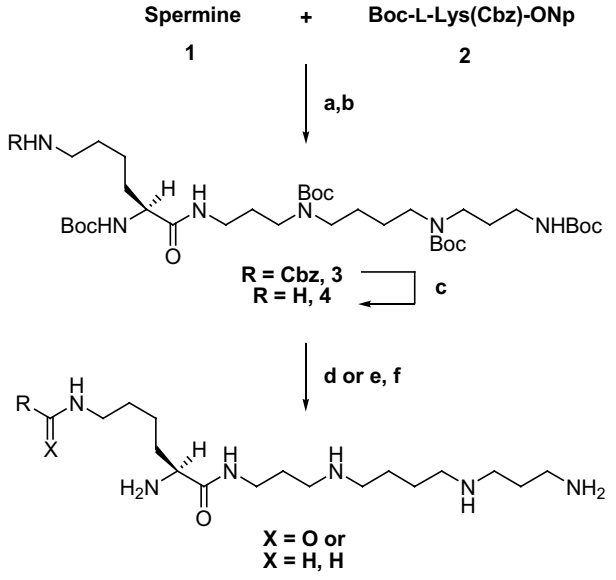


Figure 1. Structure of lipid A, the toxic moiety of bacterial lipopolysaccharide. Numbers indicate acyl chain length.



Scheme 1. Synthesis of lipophilic lysine–spermine conjugates. Conditions and reagents: (a) CH_3OH; (b) Boc_2O, THF/H_2O, Na_2CO_3; (c) H_2, Pd/C, EtOH; (d) RCOCl, Et_3N, CH_2Cl_2; (e) 1. RCHO, $CH(OCH_3)_3$, Et_3N, CH_2Cl_2, 2. $NaBH_4$, CH_3OH; (f) 3 N HCl/CH_3OH.

ine **1** with either the L- or D-stereoisomer of the orthogonally-protected, active ester Boc-Lys(Cbz)-ONp **2**. Dropwise addition of the active ester to a solution of spermine gave the statistical distribution of mono-, di-, and un-substituted products. Reaction of the remaining unsubstituted amino groups of spermine with an excess of Boc_2O produced the per-Boc mixture. The resulting mixture could now be separated by standard silica gel chromatography. The purified mono-acylated derivative **3** was then subjected to catalytic hydrogenation in order to remove the Cbz-protecting group and gain the free amino intermediate **4**. It was found to be critical to use ketone-free ethanol during this hydrogenation in order to prevent formation of a higher R_f, alkylated side-product. The amine **4** was then functionalized by standard acylation or reductive alkylation conditions to produce the protected forms of the Lys–spermine analogues. For the mono-alkyl derivatives, the imines were pre-formed then reduced using $NaBH_4$. In the case of dialkylated analogues **30** and **37**, excess aldehyde was used in a reductive amination reaction with $NaBH_3CN$. In several cases unique functional groups were synthesized using common reaction conditions or from commercial sources. The derivatized intermediates were purified using SiO_2 chromatography, and the Boc-groups removed using 3 N HCl in MeOH to afford the Lys–spermine analogues in their HCl salt forms. Compounds were characterized by TLC, ^1H and ^{13}C NMR, and LC/MS and all spectra were consistent with structures assigned.

2.2. Structure–activity relationships: binding affinity and in vitro neutralization potency

We examined the relative binding affinities of the Lys–spermine analogues with a recently-described[46] high-throughput fluorescence based displacement assay, using BODIPY-TR cadaverine (BC), and are reported as half-maximal effective displacement of probe (ED_{50}) in Figure 2, and Tables 1–3. Murine monocytes (J774A.1 cells) produce measurable quantities of NO on exposure to LPS and provide a model for the rapid assessment of compounds to neutralize LPS activity. Compounds that neutralize LPS inhibit NO production in a dose-dependant manner from which 50% inhibitory concentrations (IC_{50}) can be determined, as shown in Figure 3, and Tables 1–3. In all experiments, Polymyxin B (PMB), a decapeptide antibiotic, known to bind and neutralize LPS,[29,47,48] was used as a reference compound.

(i) *Hydrocarbon chain length.* Lysine–spermine analogues with an unsubstituted ε-amino lysine, **5** (L-Lys, ED_{50}: 40 μM), and **6** (D-Lys, ED_{50}: 58 μM) showed poor binding in the displacement assays, and negligible inhibition of LPS-induced NO production. Substitution of the ε-amino group of lysine manifests in an increase in affinity (Table 1), but no striking correlation between hydrocarbon chain-length and affinity is evident (Fig. 4, inset). In contrast, increasing carbon chain length is clearly correlated to the potency of inhibition of LPS activity (Fig. 4). We have earlier shown that this apparent discordance is attributable to the displacement of

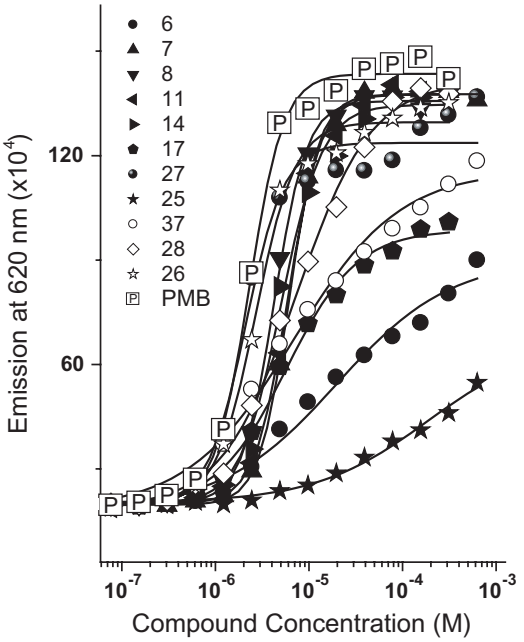
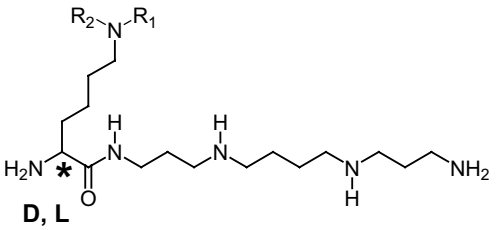


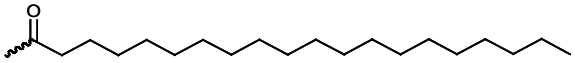
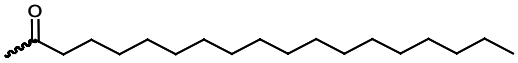
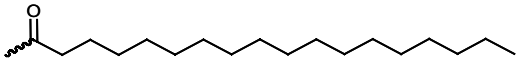
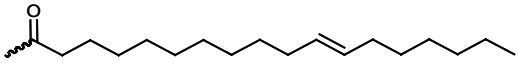
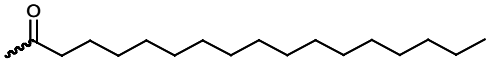
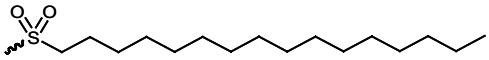
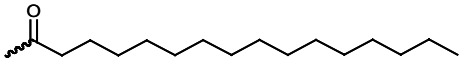
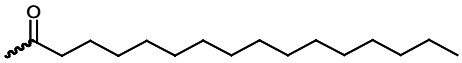
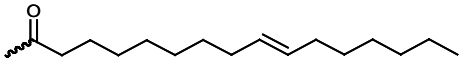
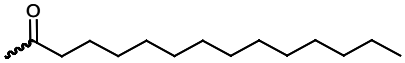
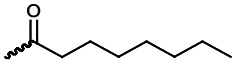
Figure 2. Binding affinity of compounds to LPS determined by the BODIPY-Cadaverine displacement method. Polymyxin B (PMB) was used as the reference compound. Alkyl compounds are depicted with open symbols and dotted lines, and acyls with closed symbols and solid lines. The Z' factor of the HTS assay for quantifying ED_{50} (relative binding affinity) is 0.82, and the CVs at 0% and 100% probe displacement are 4.1% and 6.2%, respectively.[51]

LPS-bound BC being relatively insensitive to hydrophobic substituents, and dominated by electrostatic interactions.[46] The consequence of this limitation is the lack of discrimination between ligands that merely bind LPS, and those that truly neutralize LPS activity. All promising leads are also therefore routinely screened in NO inhibition assays. Chain lengths were critical determinants for NO-inhibiting activity as shown by the alkyl homologs C_6 **31** (>1000 μM), C_7 **29** (46 μM), Δ11-C_{16} **27** (0.66 μM), as well as the acyl series from C_8 **17** (160 μM) and to C_{20} **7** (1.2 μM).

(ii) *Chain unsaturation. trans*-Unsaturation of the acyl chain was found to increased binding as shown by comparing Δ9-L-Lys-C_{16} **15** (3.8 μM) with L-Lys-C_{16} **14** (11 μM), and Δ11-L-Lys-C_{18} **10** (4.2 μM) with L-Lys-C_{18} **9** (16 μM). Similarly for the alkyls, the *cis*-unsaturated L-Lys-Δ11-C_{16} **27** displayed a higher ED_{50} of 2.6 μM compared to its saturated counterpart, C_{16} **26** (5.6 μM). However, this is not paralleled by an improvement in LPS-neutralizing activity; for instance, the fully saturated L-Lys-C_{16} **14** (IC_{50}: 6.4 μM), and the unsaturated **15** (IC_{50}: 8.8 μM) are equipotent. We surmise that the observed enhanced affinity with the unsaturated analogues may also be an artifactual consequence of the probe displacement method. The unsaturated compounds are, in general marginally more water soluble than their saturated homologs, and thus may exhibit a higher effective local concentration at the LPS-bulk solvent interface.[34] It is to be noted that in vitro bioassays, as well as in animal models, the problem of differential solubility is mitigated by the presence of physiological concentrations of albumin, which serve to solubilize

Table 1. Lysine–spermine long acyl chain homologs



ID	R_1	R_2	Note	Stereo	ED_{50} (μM)	NO IC_{50} (μM)
5	H	H		L	40.42	>1000
6	H	H		D	58.42	>1000
7		H	C_{20}	L	6.46	1.21
8		H	C_{18}	D	8.8	1.98
9		H	C_{18}	L	16.39	18.14
10		H	Δ11, C_{18}	L	4.2	NA
11		H	C_{17}	L	6.71	4.49
12		H	C_{16}	L	5.93	NA
13		H	C_{16}	D	9.94	2.29
14		H	C_{16}	L	10.74	6.41
15		H	Δ9, C_{16}	L	3.82	8.85
16		H	C_{14}	L	5.63	1.60
17		H	C_8	L	12.97	162.24

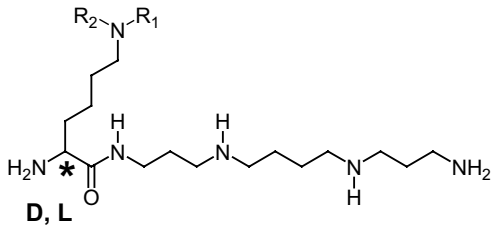
both LPS and ligand.[28] Unsaturation of the hydrophobic substituent, therefore, while not expected to result in higher potency compounds, is a potentially useful strategy that we are presently evaluating in enhancing the solubility of other, less-soluble analogues.

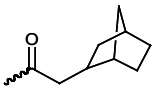
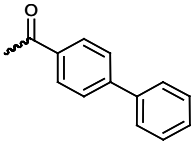
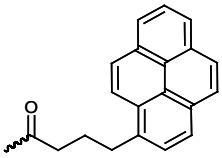
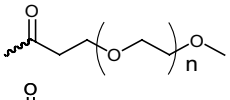
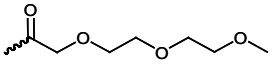
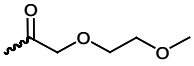
(iii) *Steric interactions.* Although analogues with short bulky substituents showed increased binding with increasing chain carbon number, for instance, comparing isobutyl **35** (101 μM) with **32** (9.6 μM) derived from (*S*)-(−)-citronellal and the bis-alkylated methylcyclohexyl **37** (4.0 μM) (Table 3), none of these compounds are potent LPS neutralizers, as are the di- and tri-ether homologs **24** and **25** (IC_{50}: >1000 mM) and the polyethylene glycol polymer **23** (320 μM). The biphenyls **38** and **21** and anthracene **22** all yielded reasonably high LPS affinities (ED_{50}: 3.7, 7.9, and 7.1 μM, respectively) but were poor inhibitors of LPS bioactivity (IC_{50}: >100 μM). These results emphasize the obligatory requirement for long-chain aliphatic hydrocarbon substituents for optimal biological potency.

(iv) *Stereochemistry of Lys residue*. Inverting the stereochemistry of the α-carbon of lysine caused no appreciable effect on binding affinity for the stereoisomeric pair D-Lys-C_{16} **13** (10 μM) and L-Lys-C_{16} **14** (11 μM), but a distinct enhancement for longer chain D-Lys-C_{18} **8** (8.8 μM), as compared to L-Lys-C_{18} **9** (16 μM). This is consistent with the higher potency for the D-analogues in inhibition NO production. The D-stereoisomers are expected to be less susceptible to proteolytic cleavage, and the higher observed activity may be a consequence of the longer persistence of the D-isomer in the assay system. It is also possible that the lipid A moiety is a chiral, and the mode of binding may be effected by the configuration of asymmetric centers. Future isothermal titration calorimetry experiments will help to clarify this issue.

(vi) *Alkylation versus acylation.* Alkyl compounds bound more strongly than their acyl equivalents; compare, for example: alkyl C_{16} **26** (5.6 μM) and acyl C_{16} **14** (11 μM). This may be attributable to the loss of a protonatable positive charge on acylating the ε-amino group, leading to poorer solubility, as mentioned earlier.

Table 2. Lysine–spermine mixed acyl analogues



ID	R_1	R_2	Note	Stereo	ED_{50} (μM)	NO IC_{50} (μM)
18		H		D	298.85	>1000
19		H		D	327.04	>1000
20		H		D	16.16	NA
21		H		D	7.86	124.30
22		H		L	7.09	108.10
23		H	Polymer	L	310.95	323.54
24		H		L	572.5	>1000
25		H		L	495.19	>1000

2.3. Comparison of IC_{50} and ED_{50}

The graph of IC_{50} versus ED_{50} values display a linear trend with a correlation coefficient of $R = 0.60$ (Fig. 5). LPS binders with strong hydrophobic interactions strayed from linearity due to the BC-LPS displacement assay not accurately predicting hydrophobic interactions, which have been shown to be crucial for LPS neutralization. This is seen also for the aromatic and bulky substituents, which are relatively bereft of biological activity in contrast to their high binding affinities and so appeared as a cluster in the upper left hand side of the IC_{50} versus ED_{50} graph (Fig. 5).

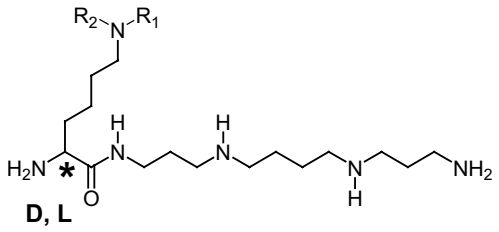
2.4. Comparison of LPS from different Gram-negative bacteria

Although the structure of lipid A is highly conserved among Gram-negative bacteria, the polysaccharide domain is highly diverse among Gram-negative bacteria.[49,50] Since the Lys–spermine library was designed to bind to the conserved lipid A portion, we expected that there would be little variation in binding to a diverse range of LPS from different bacteria. As shown in Figure 6, the highest affinity Lys–spermine analogues were shown to consistently bind to LPS from different bacteria in the 1–10 μM region and the relatively poor binders bound to all the LPS in the 10–100 μM range. This clearly shows that the Lys–spermine compounds bind to a variety of LPS structures, and thus may be clinically useful.

2.5. Dose-dependent inhibition of proinflammatory cytokines in human whole blood, determined by multiplexed cytometric bead assay

Having verified that the Lys–spermine compounds were active in inhibiting NO production in murine macrophages, we wished to independently confirm that they would also inhibit LPS-induced inflammatory responses in human cells. The activity of a subset of active Lys–spermine compounds was examined for their ability to inhibit TNF-α and IL-6 production in whole human

Table 3. Lysine–spermine mixed alkyl analogues



ID	R_1	R_2	Note	Stereo	ED_{50} (μM)	NO IC_{50} (μM)
26		H	C_{16}	L	5.56	NA
27		H	Δ11, C_{16}	L	2.59	0.66
28		H	C_7	D	3.86	>100
29		H	C_7	L	5.99	46.43
30		R_1	*bis*, C_7	L	2.14	38.83
31		H	C_6	D	7.13	>1000
32		H		D	9.55	>1000
33		H		L	12.07	>1000
34		H		D	10.93	838.41
35		H		D	100.58	72.88
36		H		D	16.08	>1000
37		R_1	*bis*	L	4.04	>1000
38		H		D	3.71	105.12

blood, stimulated ex vivo with LPS. As shown in Figure 7, the rank-order of the inhibitory potencies in this assay generally parallels NO inhibition activity, **8** being almost as potent as polymyxin B, the reference compound.

2.6. Protective effects in a mouse model of endotoxic shock

Based on the results of the displacement assays, NO, and cytokine inhibition data, **8** was elected for detailed evaluation in animal experiments. We had previously determined that the LD_{100} (lethal dose—100%) dose was 100 ng per mouse (female, outbred, CF-1 mice, sensitized with 800 mg/kg D-galactosamine). In all experiments reported in this paper, a supralethal dose of 200 ng per mouse, in a final volume of 0.2 mL saline was used. The dose–response of protection afforded by **8** is depicted in Table 4. It is noteworthy that full protection against the supralethal LPS challenge is observed at a dose of 200 μg/mouse of **8**. In these dose–response experiments, mice received graded doses of compound diluted in saline intraperitoneally (i.p.), in one flank, immediately before a supralethal (200 ng) LPS challenge, which was administered as a separate i.p. injection into the other flank. Previous studies with labile spermine conjugates such as DOSPER[37] had shown the window of protection to be very short, a 15 min window of protection. We wished to examine if **8**, with its greater anticipated hydrolytic stability, would afford a more extended time-window of protection. 200 μg of **8** in a final volume of 0.2 mL injections were administered intraperitoneally at times of −6, −4, −2, 0, +1, and +2 relative to time-zero, the time at which all mice were challenged with 200 ng/mouse LPS injections. Compound **8** provided significant protection up to 6 h prior to LPS challenge (Table 5). Based on these results, another time-course experiment with subcutaneous, rather than i.p. injections was undertaken with a much longer time window (−24, −16, −12, −8, −4, 0, and +2 h relative to the time of LPS administration). We wished to test if in this treatment regime, which is characterized by a slow, gradual systemic absorption from the site of injection, a longer duration of protection would be observed. Lethality was once again assessed 24 h following the final injection. Two of the five mice in the −24 cohort survived, as did three of the five in the −16, −12, and −8 cohorts (Table 6), indicating significant protection even when the compound is administered 16 h ahead of LPS challenge. These results indicate a significantly prolonged temporal window of protection compared to DOSPER.[37]

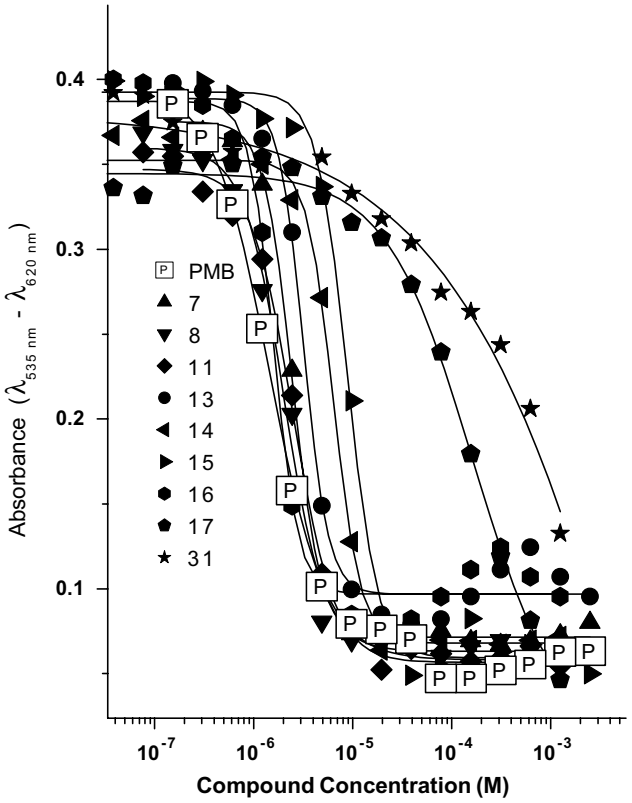


Figure 3. Nitric oxide (NO) inhibition in murine J774A.1 cells stimulated for 14 h with 10 ng/mL *E. coli* 0111:B4 LPS, by polymyxin B (reference compound), acyl (closed symbols) and alkyl (open symbols) Lys–spermine analogues. Inhibition constant (IC_{50}) values, determined from the four-parameter logistic fits, are presented in Tables 1–3. The CVs for the NO assay is 3.2%.

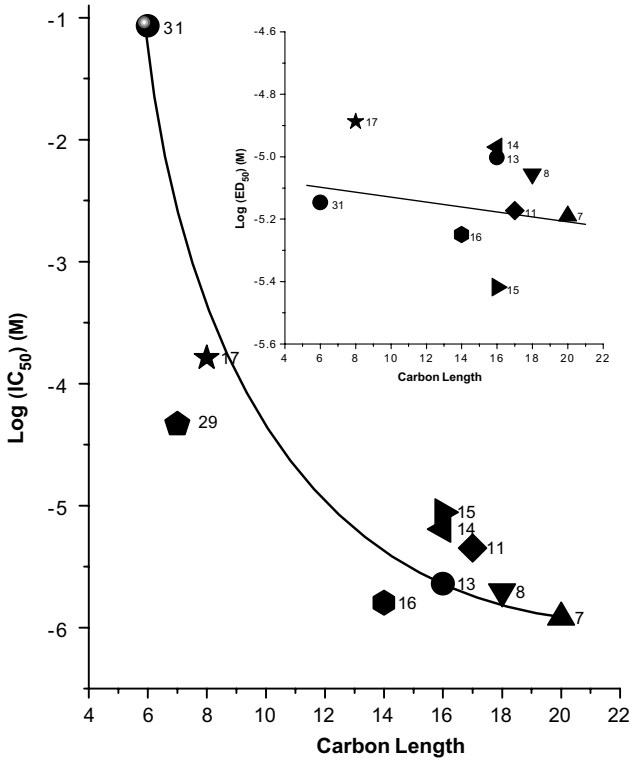


Figure 4. Correlation of NO inhibitory potency with carbon-length of straight-chain acyl/alkyl analogues. Inset: Correlation between binding affinity (BC Displacement) and carbon chain length. The poor correlation is a consequence of the relative insensitivity of the probe displacement method to discriminate between binders and true neutralizers.

3. Conclusions

In conclusion, the interactions of a focused library of lysine–spermine conjugates with Gram-negative bacterial lipopolysaccharides have been characterized. Lysine–spermine conjugates with the ε-amino terminus of the lysinyl moiety derivatized with long-chain aliphatic hydrophobic substituents in acyl or alkyl linkage bind to the lipid A moiety of LPS, and neutralize their toxicity. The presence of long-chain aliphatic hydrophobic functionalities is an obligatory requirement for biological activity. The utilization of nontoxic and ubiquitous building blocks (spermine, lysine, and long-chain fatty acid) in the synthesis of these compounds would predict low systemic toxicity, and are therefore excellent leads in the development of novel therapeutic agents aimed at the prevention or treatment of endotoxic shock states.

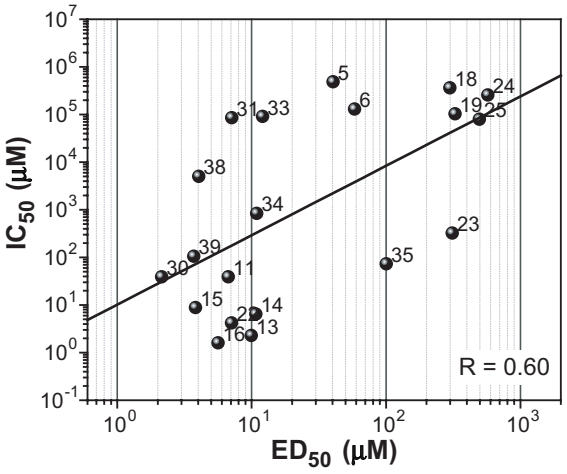


Figure 5. Correlation of binding affinity of the Lys–spermine analogues (ED_{50}) determined by BC fluorescent probe displacement, with NO inhibition (IC_{50}) in murine J774 cells.

4. Experimental

4.1. General synthetic methods

The sources of all chemical reagents and starting materials were of the highest grade available and were used without further purification. Thin-layer chromatography analysis and column chromatography were performed using Merck F_{254} silica gel plates and Baker 40 μm flash chromatography packing, respectively. TLC analysis used the following solvent systems with detection by ninhydrin staining: (a) hexane/ethyl acetate/methanol 48:48:4; (b) 2-propanol/pyridine/glacial acetic acid/H_2O, 4:1:1:2; (c) $CHCl_3$/MeOH/NH_4OH 85:15:1. LC/MS analyzes were performed using a Gilson

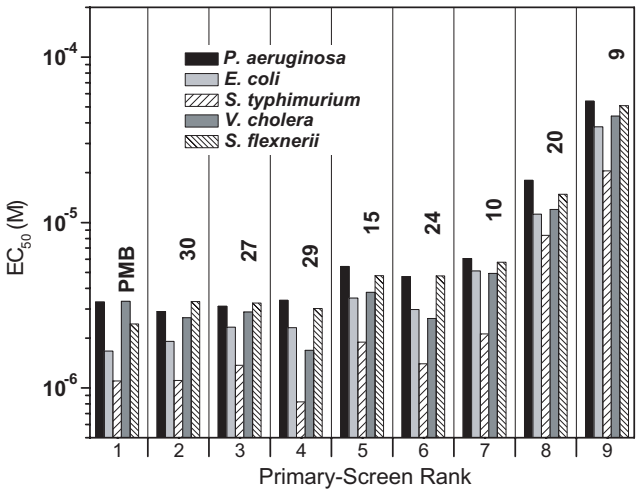


Figure 6. Lysine–spermine compounds bind to LPS isolated from diverse Gram-negative bacteria. The rank-order of binding potency is consistent.

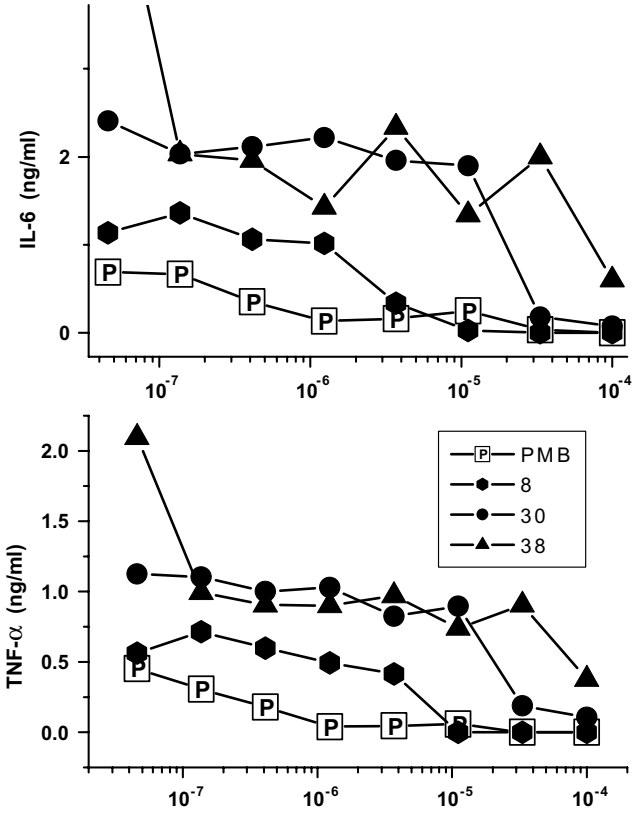


Figure 7. Inhibition by select Lys–spermine compounds of proinflammatory cytokines TNF-α and IL-6 in human blood stimulated with 10 ng/mL *E. coli* 0111:B4 LPS. Means of triplicates of a single representative experiment are shown. The CV for the cytokine measurements are 4.3%.

322 HPLC system coupled to a 215 liquid handler. Retention of these polar molecules on C-18 reverse-phase HPLC media was facilitated by the use of 0.05% heptafluorobutyric acid as an ion-pairing reagent in the mobile phase. This allowed analysis of the compounds in their underivatived forms.

Table 4. Dose-dependent protection of CF-1 mice challenged with a supralethal dose 200 ng/mouse by compound **8** in cohorts of five animals

Amount of compound used (μg/mouse)	No of live mice/no of total mice tested
0	0/5
10	0/5
50	1/5
100	4/5*
200	5/5*

Lethality was recorded at 24 h post-LPS injection. Ratios denote live/total animals. Asterisks indicate statistical significance ($P < 0.05$; Fisher one-tailed exact test).

Table 5. Time-course protection afforded by **8** in the D-galactosamine sensitized CF-1 mouse lethality model

Time of LPS administration (h)	No of live mice/no of total mice tested
−6	3/5
−4	4/5*
−2	4/5*
0	4/5*
+1	0/5*
+2	2/5

Animals were injected with 200 μg of **8** intraperitoneally at times noted with respect to LPS challenge (200 ng/mouse). Lethality was recorded at 24 h following LPS injection. Asterisks indicate statistical significance ($P < 0.05$; Fisher one-tailed exact test).

Table 6. Time-course protection afforded by compound **8** in the D-galactosamine sensitized CF-1 mouse lethality model

Time of LPS administration (h)	No. of live mice/total no. of mice tested
−24	2/5
−16	3/5
−12	3/5
−8	3/5
−4	5/5*
0	1/5
+2	1/5

Animals were injected with 200 μg of **8** subcutaneously at times noted with respect to LPS challenge (200 ng/mouse). Lethality was recorded at 24 h following LPS injection. Asterisks indicate statistical significance ($P < 0.05$; Fisher one-tailed exact test).

Detection was by a Finnigan AQA operating in ESI^+ mode (*m/z* range 140–1600 amu) together with an Agilent 1100 series DAD detector (UV range 220–320 nm). Gradient elution from 2 to 7 min. was performed using 2–100% CH_3CN in H_2O (both with 0.05% heptafluorobutyric acid added as the volatile ion-pairing reagent). ^1H and ^{13}C NMR spectra were recorded at 500 and 125.8 MHz, respectively, on a Brucker WM500 spectrometer at the University of Washington, Seattle. ^1H NMR signals were generally multiples unless otherwise noted as s = singlet, d = doublet, or t = triplet. Chemical shifts are relative to external 3-(trimethylsilyl)-1-propanesulfonic acid, sodium salt.

4.2. Synthetic methods for precursor compounds

4.2.1. Boc-L-Lys(Cbz)-N^1-spermine-Boc_3 (3). To a stirred solution of spermine **1** (11.30 g, 1.4 equiv, free base form) in MeOH (200 mL) was added dropwise over 1.5 h the active ester **2** (20.0 g, 40 mmol) in MeOH (200 mL) at room temperature. After this dropwise addition, TLC analysis (b) showed the expected mixture of products had formed (di-substituted side-product $R_f = 0.76$; mono-substituted desired product $R_f = 0.50$ and un-substituted spermine $R_f = 0.08$). If the optimal ratio was not produced additional active ester in MeOH was added dropwise. After stirring for 2 h, the solvent was evaporated to give a yellow solid that was suspended in THF (300 mL) and H_2O (100 mL). A solution of di-*tert*-butyl carbonate (43.5 g, 5.0 equiv) in tetrahydrofuran (50 mL) was added at room temperature. The pH was adjusted periodically to ~10 with a 10% Na_2CO_3 solution. A precipitate was noted after 10 min. After stirring for 18 h, TLC analysis (a) showed the expected products had formed (elution order had inverted from that given above). Most of the THF was evaporated in vacuo. The resulting mixture was dissolved in EtOAc (400 mL) and H_2O (400 mL). The organic layer was removed and the aqueous layer was re-extracted with EtOAc (3×400 mL). The combined organic layers were washed with ice-cold 0.1 N HCl (2×250 mL) followed by brine. The organic layer was dried over $MgSO_4$, filtered, and concentrated to give a crude oil, which was purified via silica gel chromatography (column dimensions 8×17 cm) using stepwise elution with 1:1 hexanes/EtOAc containing 0%, 2%, 3%, 4%, and 5% MeOH (1 L each). The order of elution is Boc_4-spermine (25% yield (spermine can be recovered after acid deprotection and conversion to the free base)), the desired mono-substituted Boc-Lys(Cbz)-spermine-Boc_3 **3** (19.4 g, 56% yield) and finally eluting last was the di-substituted side-product. ^{1}H NMR of the desired product showed this to be a mixture of *cis*- and *trans*-carbamate rotomers. It was used in the next reactions without further characterization.

4.2.2. Boc-L-Lys-N^1-spermine-Boc_3 (4). To a stirred solution of the orthogonally protected lysine–spermine conjugate **3** (19.4 g, 22.5 mmol) in EtOH (200 mL, *must use ketone and aldehyde free EtOH*!) was added palladium 10 wt % on activated carbon (10.0 g) in a round-bottom flask. The reaction flask was purged 3× with H_2 then placed under 5 psi H_2 pressure. After stirring for 4.0 h at room temperature, TLC analysis (c) showed the reaction was complete. An extra amount of activated charcoal was added to the mixture and the catalyst was removed by filtering over a pad of Celite. The pad was washed with EtOH (2×50 mL) and the combined filtrates were evaporated to give 4 as white foam in quantitative yield. Following evaluation by the above TLC system this product was used directly in the next steps.

4.3. Representative acylation reaction

4.3.1. L-Lys(palmitoyl)-N^1-spermine (14). To the amine precursor **4** (9.66 g, 13.22 mmol) was added Et_3N (5.5 mL, 3.0 equiv) and dry CH_2Cl_2 (100 mL) via syringe under an atmosphere of argon. The resulting solution was chilled to 0 °C in an ice bath and palmitoyl chloride (6.0 mL, 1.5 equiv) was added via syringe. After stirring under an argon atmosphere overnight TLC analysis (c) showed the expected product had formed. The solution was diluted in CH_2Cl_2 (100 mL) and H_2O (100 mL). The organic layer was removed and the aqueous layer was extracted twice more with CH_2Cl_2 (2×100 mL). The combined organic layer was extracted with ice cold 0.1 N HCl (100 mL) then brine and dried over $MgSO_4$, filtered, and concentrated to give the crude oil. This was purified via silica gel chromatography (column dimensions 8×17 cm) using stepwise elution with hexanes/EtOAc 1:1 containing 0%, 2%, 3%, 4%, 5%, and 6% MeOH (500 mL each) to give the Boc-protected product **13** as a clear oil (6.48 g, 51%). Removal of the protecting groups was accomplished by treating a stirred solution of the above product (6.48 g, 6.68 mmol) in MeOH (50 mL) with 6 N HCl (50 mL) at room temperature. After 3 h TLC analysis (b) showed the reaction was complete. The solvents were evaporated to give the desired product **14** in its 4HCl salt form as a white solid (4.78 g, 100%). TLC analysis (b); $R_f = 0.19$. ^{1}H NMR (D_2O, δ): 3.93 (1H), 3.45 (1H), 3.03 (13H), 2.12 (2H), 2.02 (2H), 1.85 (4H), 1.75 (s, 4H), 1.43 (4H), 1.32 (2H), 1.11 (24H), 0.72 (t, 3H). ^{13}C NMR (D_2O, ppm): 175.7, 169.8, 53.4, 46.7 (m), 45.2, 44.6, 38.8, 36.5, 36.1, 31.9, 30.4, 30.0 (m), 29.9 (m), 29.6 (m), 29.4, 28.2, 25.7, 25.5, 23.6, 22.8, 22.7, 22.3, 21.7, 13.8. LC/MS (ret time, 7.2 min), calcd for $C_{32}H_{68}N_6O_2$ *m/z* 568, obsd 569 (MH^+). Anal. Calcd for $C_{32}H_{72}Cl_4N_6O_2$: C, 53.77; H, 10.15; N, 11.76. Found: C, 53.51; H, 10.09; N, 11.51.

4.4. Representative mono-alkylation reaction

4.4.1. L-Lys(3,3-dimethyl-1-butane)-N^1-spermine (18). To 0.58 g (0.82 mmol) of amine **4** in 5 mL of dry CH_2Cl_2 under argon was added 0.27 mL (3 equiv) of trimethylorthoformate, 0.17 mL of Et_3N (1.5 equiv), and 0.31 mL (3 equiv) of 3,3-dimethylbutyraldehyde. The resulting solution was stirred at rt for 2 h when the solvents were evaporated. The oily residue was dissolved in 5 mL of CH_3OH and 70 mg (2 equiv) of $NaBH_4$ was added. After 2 h the solvent was evaporated and the residue was partitioned between 0.01 N HCl and CH_2Cl_2 (50 mL each). The aqueous part was washed with an additional portion of CH_2Cl_2 and the combined organic layers were washed with brine, dried with $MgSO_4$, and evaporated to give 0.64 g crude oil. Column chromatography used $CHCl_3$/MeOH/concd NH_4OH 96:4:0.2 to give 0.31 g (64% yield) pure protected product. This was dissolved in 3 mL of CH_3OH and treated with 3 mL of 6 N HCl at rt for 3 h. Evaporation gave 0.24 g (96% yield) of 18 as a white solid. TLC analysis (b); $R_f = 0.21$. ^{1}H NMR (D_2O, δ): 3.95 (1H), 3.31 (2H), 3.05 (14H), 2.04 (2H), 1.88 (4H), 1.71 (6H), 1.53 (2H), 1.41 (2H), 0.88 (9H). LC/MS (ret time, 6.1 min), calcd for $C_{22}H_{50}N_6O$ *m/z* 414, obsd 415 (MH^+). Anal. Calcd for $C_{22}H_{55}Cl_5N_6O \cdot 1/2H_2O$: C, 43.61; H, 9.31; N, 13.87. Found: C, 43.63; H, 9.37; N, 13.84.

4.5. Representative di-alkylation reaction

4.5.1. L-Lys-ε-(bis-(*n*-heptyl))-N^1-spermine (30). A solution containing 0.22 g (0.30 mmol) of amine **4**, 0.42 mL (3 mmol, 10 equiv) of *n*-heptanal, and 0.19 g (3 mmol, 10 equiv) of $NaBH_3CN$ in 10 mL of CH_3OH was treated with glacial HOAc (five drops). The pH was measured to be 4 by paper. Following overnight stirring the solvent was evaporated and the residue was partitioned between 1 N NaOH and CH_2Cl_2 (50 mL each). An additional CH_2Cl_2 wash of the aqueous layer was performed and the combined organic layers were washed with brine, dried over $MgSO_4$, and evaporated to give 0.33 g crude product. Column chromatography used $CHCl_3$/MeOH/concd NH_4OH (96:4:0.2) to give 0.20 g (71% yield) pure protected product. This was dissolved in 1 mL of CH_3OH and treated with 1 mL of 6 N HCl at rt for 3 h. Evaporation gave 0.11 g (73% yield) of **30** as a white solid. TLC analysis (b), $R_f = 0.21$. ^{1}H NMR (D_2O, δ): 3.93 (t, 1H), 3.28 (2H), 3.04 (16H), 2.02 (2H), 1.85 (4H), 1.71 (s, 4H), 1.62 (6H), 1.40 (4H), 1.25 (14H), 0.81 (t, 6H). ^{13}C NMR (D_2O, ppm): 175.7, 169.8, 53.4, 46.7 (m), 45.2, 44.6, 38.8, 36.5, 36.1, 31.9, 30.4, 30.0 (m), 29.9 (m), 29.6 (m), 29.4, 28.2, 25.7, 25.5, 23.6, 22.8, 22.7, 22.3, 21.7, 13.8. LC/MS (ret time, 7.3 min), calcd for $C_{32}H_{68}N_6O_2$ *m/z* 568, obsd 569 (MH^+).

4.6. Representative individual analogues

4.6.1. L-Lys-N^1-spermine (5).[1] TLC analysis (b); $R_f = 0.04$. LC/MS (ret time, 5.5 min), calcd for $C_{16}H_{38}N_6O$ *m/z* 330, obsd 331 (MH^+).

4.6.2. D-Lys-N^1-spermine (6). Synthesis of analogue **6** used Boc-D-Lys(Boc)-ONp in place of the orthogonally protected lysine derivative used for the synthesis of **14**. Coupling with spermine followed by protection of the remaining amino groups as their Boc-carbamates gave the protected intermediate following purification by column chromatography. Deprotection using 6 N HCl in CH_3OH gave the desired product **6**. TLC analysis (b); $R_f = 0.04$. ^{1}H NMR (D_2O, δ): 3.92 (t, 1H), 3.29 (2H), 3.07 (10H), 2.93 (t, 2H), 2.04 (2H), 1.84 (4H), 1.72 (4H), 1.54 (2H), 1.34 (2H). ^{13}C NMR (D_2O, ppm): 168.7, 52.2, 45.8 (m), 44.2, 43.6, 40.0, 37.8, 35.4 (m), 29.0, 25.4, 24.6, 22.6, 21.8 (m), 20.2. LC/MS (ret time, 5.5 min), calcd for $C_{16}H_{38}N_6O$ *m/z* 330, obsd 331 (MH^+). HRMS *m/z* calcd for $C_{16}H_{38}N_6O$ (M + H) 331.3185, found 331.3173. Anal. Calcd for $C_{16}H_{43}Cl_5N_6O{\cdot}3/2H_2O$: C, 35.60; H, 8.59; N, 15.57. Found: C, 35.58; H, 8.47; N, 15.41.

4.6.3. L-Lys-ε-(eicosanoyl)-N^1-spermine (7). TLC analysis (b); $R_f = 0.08$. ^{1}H NMR (D_2O, δ): 3.94 (1H), 3.48 (1H), 3.06 (13H), 2.15 (2H), 2.06 (2H), 1.88 (4H), 1.75 (4H), 1.47 (4H), 1.36 (2H), 1.16 (32H), 0.77 (3H). ^{13}C NMR (D_2O, ppm): 175.5, 169.8, 53.4, 46.9 (m), 45.6, 44.8, 38.8, 36.8 (m), 36.0, 31.9, 30.4, 29.7 (m), 29.5, 29.3, 28.5, 25.9, 25.7, 23.8, 23.2, 23.1, 22.8, 21.7, 13.8. LC/MS (ret time, 7.6 min), calcd for $C_{36}H_{76}N_6O_2$ *m/z* 625, obsd 626 (MH^+).

4.6.4. D-Lys-ε-(stearoyl)-N^1-spermine (8). TLC analysis (b); $R_f = 0.13$. ^{1}H NMR (D_2O, δ): 3.94 (1H), 3.47 (1H), 3.06 (13H), 2.13 (2H), 2.04 (2H), 1.87 (4H), 1.75 (4H), 1.47 (4H), 1.36 (2H), 1.16 (28H), 0.79 (3H). ^{13}C NMR (D_2O, ppm): 175.9, 170.1, 53.4, 47.1 (m), 45.5, 44.7, 39.0, 36.8 (m), 36.0, 31.9, 30.6, 29.8 (m), 29.6, 29.3, 28.4, 25.9, 25.7, 23.8, 23.2, 23.1, 22.8, 13.8. LC/MS (ret time, 7.4 min), calcd for $C_{32}H_{68}N_6O_2$ *m/z* 597, obsd 598 (MH^+).

4.6.5. L-Lys-ε-(stearoyl)-N^1-spermine (9). LC/MS (ret time, 7.4 min), calcd for $C_{34}H_{72}N_6O_2$ *m/z* 597, obsd 598 (MH^+).

4.6.6. L-Lys-ε-(heptadecanoyl)-N^1-spermine (11). TLC analysis (b); $R_f = 0.19$. ^{1}H NMR (D_2O, δ): 3.96 (1H), 3.47 (1H), 3.08 (13H), 2.14 (2H), 2.04 (2H), 1.87 (4H), 1.78 (4H), 1.50 (4H), 1.36 (2H), 1.22 (26H), 0.78 (3H). ^{13}C NMR (D_2O, ppm): 175.7, 169.9, 53.2, 47.2 (m), 45.4, 44.8, 39.0, 36.6 (m), 36.1, 31.9, 29.9 (m), 29.5, 29.3, 28.4, 25.8, 25.7, 23.8, 22.6, 22.5, 22.2, 22.0, 14.8. LC/MS (ret time, 7.2 min), calcd for $C_{33}H_{70}N_6O_2$ *m/z* 583, obsd 584 (MH^+).

4.6.7. L-Lys-ε-(hexadecanesulfonamide)-N^1-spermine (12). ^{1}H NMR (D_2O, δ): 4.04 (1H), 3.53 (1H), 3.30 (1H), 3.22 (2H), 3.17 (14H), 2.18 (2H), 2.00 (4H), 1.82 (6H), 1.67 (2H), 1.52 (4H), 1.34 (22H), 0.95 (t, 3H). LC/MS (ret time, 7.3 min), calcd for $C_{32}H_{70}N_6O_3S$ *m/z* 619, obsd 620 (MH^+).

4.6.8. D-Lys-ε-(palmitoyl)-N^1-spermine (13). TLC analysis (b); $R_f = 0.21$. ^{1}H NMR (D_2O, δ): 3.94 (1H), 3.47 (1H), 3.06 (13H), 2.13 (2H), 2.04 (2H), 1.87 (4H), 1.75 (4H), 1.47 (4H), 1.36 (2H), 1.16 (24H), 0.78 (3H). ^{13}C NMR (D_2O, ppm): 175.7, 169.8, 53.4, 47.2 (m), 45.6, 44.8, 39.0, 36.6 (m), 36.1, 31.9, 29.8 (m), 29.6, 29.3, 28.4, 25.9, 25.7, 23.8, 22.8, 23.1, 22.8, 22.1, 14.0. LC/MS (ret time, 7.2 min), calcd for $C_{32}H_{68}N_6O_2$ *m/z* 569, obsd 570 (MH^+).

4.6.9. L-Lys(ene-Δ9-palmitoyl)-N^1-spermine (15). LC/MS (ret time, 7.3 min), calcd for $C_{32}H_{66}N_6O_2$ *m/z* 566, obsd 567 (MH^+). ^{1}H NMR (D_2O, δ): 5.29 (2H), 3.96 (1H), 3.49 (1H), 3.08 (14H), 2.16 (2H), 2.07 (2H), 1.87 (6H), 1.75 (4H), 1.47 (4H), 1.38 (2H), 1.16 (17H), 0.78 (3H). Anal. Calcd for $C_{32}H_{70}Cl_4N_6O_2{\cdot}2H_2O$: C, 51.33; H, 9.96; N, 11.22. Found: C, 51.30; H, 9.60; N, 11.45.

4.6.10. L-Lys-ε-(myristoyl)-N^1-spermine (16). TLC analysis (b); $R_f = 0.22$. ^{1}H NMR (D_2O, δ): 3.92 (1H), 3.27 (2H), 3.03 (14H), 2.12 (2H), 2.07 (4H), 1.83 (4H), 1.66 (6H), 1.48 (4H), 1.20 (20H), 0.78 (3H). LC/MS (ret time, 7.0 min), calcd for $C_{30}H_{64}N_6O_2$ *m/z* 541, obsd 542 (MH^+).

4.6.11. L-Lys-ε-(octanoyl)-N^1-spermine (17). TLC analysis (b); $R_f = 0.20$. LC/MS (ret time, 5.7 min), calcd for $C_{21}H_{46}N_6O_2$ *m/z* 414, obsd 415 (MH^+). Anal. Calcd for $C_{24}H_{56}Cl_4N_6O_2{\cdot}H_2O$: C, 46.45; H, 9.42; N, 13.54. Found: C, 46.36; H, 9.39; N, 13.49.

4.6.12. D-Lys-ε-(isopropoyl)-N^1-spermine (18). TLC analysis (b); $R_f = 0.24$. ^{1}H NMR (D_2O, δ): 3.90 (1H), 3.28 (3H), 3.05 (13H), 2.40 (1H), 2.02 (2H), 1.82 (4H), 1.71 (s, 2H), 1.47 (2H), 1.28 (2H), 0.99 (6H). ^{13}C NMR (D_2O, ppm): 180.8, 175.8, 53.2, 47.0 (m), 45.2, 44.6, 38.6, 36.4 (m), 35.1, 30.4, 28.1, 25.7, 23.8, 29.4, 22.8 (m), 21.6, 18.9. LC/MS (ret time, 5.5 min), calcd for $C_{20}H_{44}N_6O_2$ *m/z* 400, obsd 401 (MH^+).

4.6.13. D-Lys-ε-(2-norbornaneacetoyl)-N^1-spermine (20). TLC analysis (b); $R_f = 0.22$. ^{1}H NMR (D_2O, δ): 3.88 (1H), 3.24 (2H), 3.05 (13H), 2.02 (4H), 1.80 (4H), 1.68 (4H), 1.33 (8H), 0.98 (5H). LC/MS (ret time, 6.0 min), calcd for $C_{25}H_{50}N_6O_2$ *m/z* 466, obsd 467 (MH^+).

4.6.14. D-Lys-ε-(4-biphenycarboxamide)-N^1-spermine (21). TLC analysis (b); $R_f = 0.13$. ^{1}H NMR (D_2O, δ): 7.77 (6H), 7.43 (3H), 3.87 (1H), 3.48 (2H), 3.16 (2H), 2.95 (10H), 1.94 (2H), 1.83 (2H), 1.72 (2H), 1.62 (6H), 1.34 (2H). LC/MS (ret time, 6.3 min), calcd for $C_{29}H_{46}N_6O_2$ *m/z* 511, obsd 512 (MH^+).

4.6.15. L-Lys-ε-(4-(1-pyrene)-butanoyl)-N^1-spermine (22). Synthesis of analogue **22** was by acylation with 1-pyrenebutanoic acid succinimidyl ester from Molecular Probes, Eugene, OR (cat. no. P-130). TLC analysis (b); $R_f = 0.15$. ^{1}H NMR (D_2O, δ): 7.34 (d, 1H), 7.22 (3H), 7.08 (2H), 6.98 (2H), 6.88 (d, 1H), 3.74 (t, 1H), 3.18 (1H), 3.01 (4H), 2.93 (2H), 2.86 (1H), 2.77 (1H), 2.72 (2H), 2.65 (2H), 2.56 (1H), 2.40 (2H), 1.97 (2H), 1.73 (2H), 1.68 (2H), 1.54 (6H), 1.40 (2H), 0.98 (4H). LC/MS (ret time, 6.6 min), calcd for $C_{36}H_{52}N_6O_2$ *m/z* 601, obsd 602 (MH^+).

4.6.16. L-Lys-ε-(methylpolyethyleneglycolpropionyl)-N^1-spermine (23). The active ester used to acylate the ε-nitrogen atom was mPEG-SPA (mw 2000) from Nektar Therapeutics (cat. no. 2M4MODO1). TLC analysis (b); $R_f = 0.24$. ^{1}H NMR (D_2O, δ): 3.80 (1H), 3.50 (large OCH_2 envelope), 3.42 (6H), 2.92 (15H), 2.34 (1H), 1.96 (1H), 1.75 (4H), 1.62 (4H), 1.41 (1H), 1.23 (1H). LC/MS (ret time, 6.1 min), obsd an envelope of *m/z* centered at 650.

4.6.17. L-Lys-ε-(2-[2-(2-methoxyethoxy)ethoxy]acetoyl)-N^1-spermine (24). TLC analysis (b); $R_f = 0.11$. ^{1}H NMR (D_2O, δ): 4.01 (3H), 3.91 (1H), 3.62 (6H), 3.31 (8H), 3.03 (12H), 2.06 (2H), 1.82 (6H), 1.53 (1H), 1.32 (1H). LC/MS (ret time, 5.4 min), calcd for $C_{23}H_{50}N_6O_5$ *m/z* 490, obsd 491 (MH^+).

4.6.18. L-Lys-ε-(2-(2-methoxyethoxy)acetoyl)-N^1-spermine (25). TLC analysis (b); $R_f = 0.09$. ^{1}H NMR (D_2O, δ): 3.98 (3H), 3.92 (1H), 3.62 (6H), 3.31 (6H), 3.24 (6H), 3.03 (6H), 2.06 (2H), 1.87 (2H), 1.80 (4H), 1.53 (1H), 1.32 (1H). LC/MS (ret time, 5.7 min), calcd for $C_{21}H_{46}N_6O_4$ *m/z* 446, obsd 447 (MH^+).

4.6.19. L-Lys-ε-(^{n}hexadecyl)-N^1-spermine (26). TLC analysis (b); $R_f = 0.11$. ^{1}H NMR (D_2O, δ): 3.97 (1H), 3.48 (1H), 3.04 (15H), 2.04 (2H), 1.91 (4H), 1.75 (8H), 1.48 (2H), 1.22 (26H), 0.91 (3H). ^{13}C NMR (D_2O, ppm): 168.8, 53.4, 48.0, 47.3, 47.1 (m), 45.4, 44.7, 36.7, 32.0, 30.6, 29.9 (m), 29.8, 29.5, 29.4, 29.1, 26.5, 25.9, 25.6, 25.5, 23.8, 22.9, 22.8, 21.7, 13.9. LC/MS (ret time, 7.2 min), calcd for $C_{32}H_{70}N_6O$ *m/z* 555, obsd 556 (MH^+). Anal. Calcd for $C_{32}H_{75}Cl_5N_6O{\cdot}3/2H_2O$: C, 50.29; H, 10.29; N, 11.00. Found: C, 50.30; H, 10.05; N, 10.67.

4.6.20. D-Lys-ε-(3,3-dimethyl-1-butyl)-N^1-spermine (34). TLC analysis (b); $R_f = 0.06$. ^{1}H NMR (D_2O, δ): 3.91 (1H), 3.33 (1H), 3.23 (1H), 3.05 (14H), 2.01 (2H), 1.86 (4H), 1.71 (4H), 1.67 (2H), 1.50 (2H), 1.38 (2H), 0.85 (9H). LC/MS (ret time, 6.1 min), calcd for $C_{22}H_{50}N_6O$ *m/z* 414, obsd 415 (MH^+).

4.6.21. D-Lys-ε-(3-methylpropyl)-N^1-spermine (35). TLC analysis (b); $R_f = 0.06$. ^{1}H NMR (D_2O, δ): 3.90 (1H), 3.32 (1H), 3.24 (1H), 3.05 (10H), 2.82 (2H), 2.02 (2H), 1.82 (6H), 1.71 (6H), 1.37 (1H), 0.90 (6H). LC/MS (ret time, 5.8 min), calcd for $C_{20}H_{46}N_6O$ *m/z* 387, obsd 388 (MH^+).

4.6.22. L-Lys-ε-(bis-(cyclohexyl))-N^1-spermine (37). TLC analysis (b); $R_f = 0.22$. ^{1}H NMR (D_2O, δ): 3.96 (1H), 3.30 (2H), 3.04 (18H), 2.06 (2H), 1.86 (4H), 1.72 (16H), 1.40 (2H), 1.18 (6H), 0.97 (4H). ^{13}C NMR (D_2O, ppm): 169.8, 60.2, 54.1, 53.2, 47.0 (m), 45.3, 44.6, 36.6 (m), 32.9, 30.3 (m), 25.4, 25.0, 23.8, 22.8, 22.3, 21.7. LC/MS (ret time, 6.4 min), calcd for $C_{30}H_{62}N_6O$ *m/z* 523, obsd 524 (MH^+).

4.6.23. D-Lys-ε-(4-phenylbenzyl)-N^1-spermine (38). TLC analysis (b); $R_f = 0.11$. ^{1}H NMR (D_2O, δ): 7.55 (9H), 4.21 (s, 2H), 3.93 (1H), 3.32 (1H), 3.24 (1H), 3.04 (12H), 2.04 (2H), 1.80 (4H), 1.72 (6H), 1.40 (2H). ^{13}C NMR (D_2O, ppm): 169.8, 141.6, 139.6, 130.5, 139.9, 129.3, 128.2, 127.6, 127.0, 53.1, 50.6, 47.1, 47.0, 46.5, 45.3, 44.6, 36.6 (m), 30.3, 25.5, 25.1, 23.8, 22.9 (m), 21.6. LC/MS (ret time, 6.3 min), calcd for $C_{29}H_{48}N_6O$ *m/z* 497, obsd 498 (MH^+).

4.7. Rapid-throughput fluorescence displacement assay for quantifying binding affinities to LPS

The BODIPY-TR-cadaverine (BC; 5-(((4-(4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene-3-yl) phenoxy)acetyl)amino)pentylamine, hydrochloride; obtained from Molecular probes, Inc., Eugene, OR) displacement assay to quantify the affinities of binding of compounds to LPS has been described in detail recently.[46] This assay was performed in a rapid-throughput format as follows. The first column (16 wells) of a Corning Nonbinding Surface 384-well flat-bottom black fluorescence microplate contained 15 test compounds plus polymyxin B, all at 5 mM, and were serially twofold diluted across the remaining 23 columns, achieving a final dilution of 0.596 nM in a volume of 40 μL. Polymyxin B (PMB), a peptide antibiotic known to bind and neutralize LPS[47] served as the positive control and reference compound for every plate, enabling the quantitative assessment of repeatability and reproducibility (CV and Z' factors) for the assay. The Z' factor of the HTS assay for quantifying ED_{50} (relative binding affinity) is 0.82, and the CVs at 0% and 100% probe displacement are

4.1% and 6.2%, respectively.[51] Robotic liquid handling was performed on a Precision 2000 automated microplate pipetting system, programmed using the Precision Power software, Bio-Tek Instruments Inc., VT, USA. Stock solutions of LPS (5 mg/mL; *E. coli* 0111:B4; procured from Sigma) and BC (500 μM) were prepared in Tris buffer (pH 7.4, 50 mM). One milliliter each of the LPS and BC stocks were mixed and diluted in Tris buffer to a final volume of 100 mL, yielding final concentrations of 50 μg/mL of LPS and 5 μM BC. Forty microliters of this BC:LPS mixture was added to each well of the plate using the Precision 2000. Fluorescence measurements were made at 25 °C on a Fluoromax-3 with Micromax Microwell 384-well plate reader, using DataMax software, Jobin Yvon Inc., NJ. The BC excitation wavelength was 580 nm, emission spectra were taken at 620 nm with both emission and excitation monochromator bandpasses set at 5 nm. The fluorescence of BC is quenched upon binding to LPS, and the displacement of BC by the compounds results in de-quenching (intensity enhancement) of BC fluorescence. Effective displacements (ED_{50}) were computed at the midpoint of the fluorescence signal versus compound concentration displacement curve, determined using an automated four-parameter sigmoidal fit utility of the Origin plotting software (Origin Lab Corp., MA), as described in the preceding paper. Z' factors[52] computed using the equation: $1 - [3(SD + SD')/(A - A')]$ where SD and SD′, A and A' are standard deviations for the signal and noise, and means of signal and noise, respectively, yielded a Z' factor of 0.821 and an inter-plate CVs of 5.2%.

4.8. Nitric oxide assay

Nitric oxide production was measured as total nitrite in murine macrophage J774A.1 cells using the Griess reagent system.[53,54] Murine macrophage J774A.1 cells were grown in RPMI-1640 cell-culture medium containing L-glutamine and sodium bicarbonate and supplemented with 10% fetal bovine serum, 1% L-glutamine–penicillin–streptomycin solution, and 200 μg/mL L-arginine at 37 °C in a 5% CO_2 atmosphere. Cells were plated at $\sim 2 \times 10^6$/mL in a volume of 40 μL/well, in 384 well, flat-bottomed, cell culture treated microtiter plates until confluency and subsequently stimulated with 100 ng/mL lipopolysaccharide (LPS). Concurrent to LPS stimulation, serially diluted concentrations of test compounds were added to the cell medium and left to incubate overnight for 16 h. Polymyxin B was used as reference compound in each plate. Positive- (LPS stimulation only) and negative-controls (J774.A1 medium only) were included in each experiment. Nitrite concentrations were measured adding 30 μL of supernatant to equal volumes of Griess reagents (50 μL/well; 0.1% NED solution in ddH_2O and 1% sulfanilamide, 5% phosphoric acid solution in ddH_2O) and incubating for 15 min at room temperature in the dark. Absorbance at 535 nm was measured using a Molecular Devices Spectramax M2 multifunction plate reader (Sunnyvale, CA). Nitrite concentrations were interpolated from standard curves obtained from serially diluted sodium nitrite standards.

4.9. Multiplexed cytokine assay ex vivo in human blood

One hundred microliters aliquots of fresh whole blood, anticoagulated with EDTA, obtained by venipuncture from healthy human volunteers with informed consent and as per guidelines approved by the Human Subjects Experimentation Committee, were exposed to an equal volume of 50 ng/mL of *E. coli* 0111:B4 LPS, with graded concentrations of test compounds diluted in saline for 4 h in a 96-well microtiter plate. The effect of the compounds on modulating cytokine production examined using a FACSArray multiplexed flow-cytometric bead array (CBA) system (Becton–Dickinson–Pharmingen, San Jose, CA). The system uses a sandwich ELISA-on-a-bead principle,[55,56] and is comprised of 6 populations of microbeads that are spectrally unique in terms of their intrinsic fluorescence emission intensities (detected in the FL3 channel of a standard flow cytometer). Each bead population is coated with a distinct capture antibody to detect six different cytokines concurrently from biological samples (the human inflammation CBA kit includes TNF-α, IL-1β, IL-6, IL-8, IL-10, and IL-12p70). The beads are incubated with 30 μL of sample, and the cytokines of interest are first captured on the bead. After washing the beads, a mixture of optimally paired second antibodies conjugated to phycoerythrin is added, which then forms a fluorescent ternary complex with the immobilized cytokine, the intensity (measured in the FL2 channel) of which is proportional to the cytokine concentration on the bead. The assay was performed according to protocols provided by the vendor. Standard curves were generated using recombinant cytokines provided in the kit. The data were analyzed in the CBA software suite, which is integral to the FACSArray system.

4.10. Mouse lethality experiments

Female, outbred, 9–11-week-old CF-1 mice (Charles River, Wilmington, MA) weighing 22–28 g were used as described elsewhere.[37] Upon arrival, the mice were allowed to acclimatize for a week prior to experimentation, housed 5 per cage in a controlled environment at the AALAC-accredited University of Kansas Animal Care Facility, and allowed access to mouse chow and water *ad libitum*. The animals were sensitized to the lethal effects of LPS by D-galactosamine. [55,57,58] The lethal dose causing 100% mortality (LD_{100}) dose of the batch of LPS used (*E. coli* 0111:B4 procured from Sigma) was first determined by administering D-galactosamine (800 mg/kg) and LPS (0, 10, 20, 50, 100, 200 ng/mouse) as a single injection intraperitoneally (i.p.) in freshly prepared saline to batches of five animals in a volume of 0.2 mL. The expected dose–response profile was observed in two independent experiments with all five mice receiving 100 ng succumbing within 24 h, establishing the LD_{100} dose to be 100 ng/mouse. Experiments designed to test dose–response effects of the acylspermines in affording protection against LPS-induced lethality, mice received graded doses of compound diluted in saline, i.p., in one flank, immediately before a supralethal (200 ng) LPS challenge, which was administered as a separate i.p. injection into the other flank. In

experiments in which the temporal window of protection was to be examined, a fixed dose of 200 μg/mouse of compound was administered at various times, before, or after supralethal (200 ng/mouse) LPS challenge. Lethality was determined at 24 h post LPS challenge.

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