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Structure-Activity Relationships of Antimicrobial and Lipoteichoic Acid-Sequestering Properties in Polyamine Sulfonamides▽

Hemamali J. Warshakoon,[1] Mark R. Burns,[2]* and Sunil A. David[1]*

Department of Medicinal Chemistry, University of Kansas, Lawrence, Kansas 66045,[1] and MediQuest Therapeutics, Inc., 22322 20th Ave. SE, Bothell, Washington 98021[2]

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We have recently confirmed that lipoteichoic acid (LTA), a major constituent of the gram-positive bacterial surface, is the endotoxin of gram-positive bacteria that induces proinflammatory molecules in a Toll-like receptor 2 (TLR2)-dependent manner. LTA is an anionic amphipath whose physicochemical properties are similar to those of lipopolysaccharide (LPS), which is found on the outer leaflet of the outer membranes of gram-negative organisms. 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 do bind to and neutralize LTA, as reflected by the inhibition of TLR2-mediated NF-κB 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. We then sought to examine possible correlations between the neutralizing potency toward LTA and antimicrobial activity in *Staphylococcus 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. 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 antisepsis and antibacterial properties.

Sepsis and its sequel, septic shock, a consequence of systemic inflammation that leads to multiple-organ failure (32), are common and serious clinical problems for which no specific therapeutic options are yet available. Sepsis is the number-one cause of death in noncardiac intensive care units (20) and accounts for some 200,000 fatalities in the United States annually (8), and the incidence continues to rise in the United States (34) and worldwide (38), despite great strides in antimicrobial chemotherapy.

The primary trigger in the septic shock syndrome caused by gram-negative bacteria is thought to be endotoxin, a constituent of the outer membrane of enterobacterial gram-negative bacteria. Endotoxins consist of a polysaccharide portion and a hydrophobic moiety called lipid A (Fig. 1) and are therefore also called lipopolysaccharides (LPSs). Total synthesis of the structurally highly conserved lipid A portion led to the demonstration that it is the active moiety of LPS (18, 27). Septic shock, however, is by no means an exclusive sequel of systemic infections caused by gram-negative bacteria (4). Owing to the increasing prevalence of nosocomial infections due to invasive procedures, immunosuppression, and cancer chemotherapy, the incidence of septic shock due to gram-positive organisms is on the rise (26, 29, 40) and is of particular concern in individuals who have neutropenia, a frequent attendant of ablative chemotherapy and radiotherapy (42). Because the shock state in systemic sepsis caused by gram-positive bacteria is clinically indistinguishable from that caused by gram-negative bacteria (30), it has generally been regarded that the initiation and progression of the systemic inflammatory response are pathophysiologically similar, regardless of the causative organism. The prominent role of LPS in the pathogenesis of shock caused by gram-negative bacteria renders lipid A a logical therapeutic target in the development of antiendotoxin strategies, and we have made considerable progress in the structure-based design and development of LPS sequestrants (10, 35, 39, 45). Unlike gram-negative bacteria, which bear LPS on the outer leaflet of the outer membrane, the external surface of the peptidoglycan layer is decorated with lipoteichoic acids (LTAs) (Fig. 1) in gram-positive organisms (1, 9). LTAs are anchored in the peptidoglycan substratum via a diacylglycerol moiety and bear a surface-exposed, polyanionic, 1-3-linked polyglycerophosphate appendage (14) which varies in its subunit composition in LTAs from various gram-positive

* Corresponding author. Mailing address for Sunil A. David: Multidisciplinary Research Building, Room 320D, 2030 Becker Drive, Lawrence KS 66047. Phone: (785) 864-1610. Fax: (785) 864-1920. E-mail: sdavid@ku.edu. Mailing address for Mark R. Burns: MediQuest Therapeutics, Inc., 22322 20th Ave. SE, Bothell, WA 98021. Phone: (425) 398-9580, ext 15. Fax: (425) 398-9590. E-mail: markburns@mqti.com.

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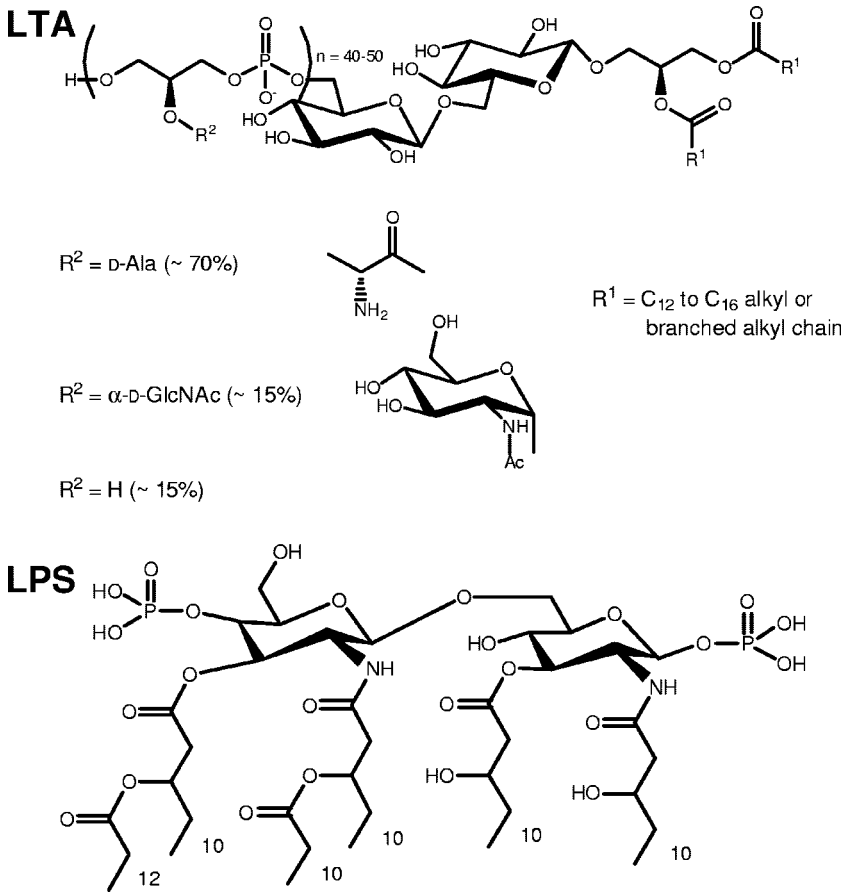


FIG. 1. Structures of LTA and LPS showing physicochemical similarities (anionic amphipathic molecules).

bacteria; in *Staphylococcus aureus*, the repeating subunit contains D-alanine and α-D-*N*-acetylglucosamine (Fig. 1) (17). There had been considerable disagreement as to the identity of the endotoxin from gram-positive organisms (22–25), but recent total syntheses of LTA species and the demonstration of LPS-like activities in synthetic LTA (13, 16, 37, 46) have served to establish that LTA stimulates the production of proinflammatory mediators. We have recently verified that the major immunostimulatory and proinflammatory component(s) of the gram-positive bacterial envelope in the human system is indeed LTA (28).

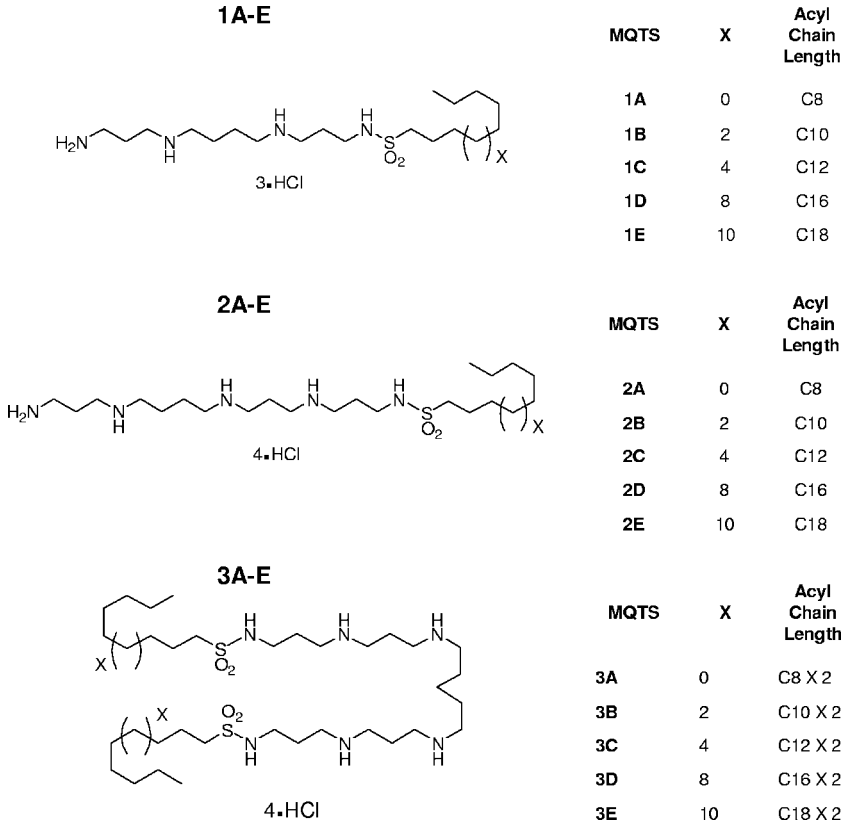
Because LTA, like LPS, is an anionic, amphipathic molecule (Fig. 1) with a hydrophobic diacylglycerol moiety conjugated to the hydrophilic and polyanionic teichoic acid (substituted polyphosphoglycerate) appendage, it was of interest to examine if our ongoing efforts at refining polycationic amphipaths designed to bind to and sequester LPS (10, 35, 39, 45) would offer leads in the design of LTA-neutralizing compounds. The hypothesis that compounds that bind to LPS as well as LTA could be identified and refined seemed particularly plausible since we had earlier found that certain compounds were potently antibacterial against both gram-negative and gram-positive species (2), suggesting that these compounds interacted with and destabilized the cellular envelopes of both types of microorganisms. We now report that the proinflammatory activities of LTA can be inhibited by sequestering LTA using compounds previously shown to be LPS-binding and -neutralizing agents. The structure-activity relationships for these compounds show a clear parallel between LTA- and LPS-sequestering activities. These results suggest that endotoxins from both gram-negative and gram-positive bacteria may be amenable to neutralization by such compounds.

MATERIALS AND METHODS

Reagents. LTA from *S. aureus*, extracted by the *n*-butanol procedure (36) and purified by delipidation and enzymatic deproteination, was procured from InvivoGen (San Diego, CA). LTAs from other commercial sources were found to be contaminated with trace quantities of Toll-like receptor 4 (TLR4)-agonistic species (probably LPS) and were not used. Highly purified LPS isolated from *Escherichia coli* O111:B4 was from List Biologicals (Campbell, CA). The syntheses and characterization of the polyamine sulfonamides (Fig. 2) have been reported earlier (5).

Cell lines. HEK-Blue-4 cells (HEK293 cells stably transfected with TLR4, MD2, and CD14, as well as secreted alkaline phosphatase [sAP] under the control of a promoter inducible by NF-κB and activator protein 1 [AP-1]) and HEK-Blue-2 cells (HEK293 cells stably transfected with TLR2, MD2, CD14, and sAP, also under the control of a promoter inducible by NF-κB and AP-1) were from InvivoGen and were maintained in HEK-Blue selection medium containing zeocin, blasticidin, and normocin, according to the vendor-supplied protocols.

NF-κB induction. The induction of NF-κB was quantified with HEK-Blue cells, as reported earlier (28). The stable expression of sAP under the control of NF-κB and AP-1 promoters is inducible by the occupancy of TLR2 (LTA) or TLR4 (LPS), and the amount of extracellular sAP in the supernatant is proportional to the level of NF-κB induction. HEK-Blue-2 or KEK-Blue-4 cells were



MQTS	X	Acyl Chain Length
1A	0	C8
1B	2	C10
1C	4	C12
1D	8	C16
1E	10	C18

MQTS	X	Acyl Chain Length
2A	0	C8
2B	2	C10
2C	4	C12
2D	8	C16
2E	10	C18

MQTS	X	Acyl Chain Length
3A	0	C8 X 2
3B	2	C10 X 2
3C	4	C12 X 2
3D	8	C16 X 2
3E	10	C18 X 2

FIG. 2. Structures of the polyamine sulfonamides.

incubated at a density of $\sim 10^5$ cells/ml in a volume of 80 μl/well in 384-well, flat-bottomed, cell culture-treated microtiter plates until confluence was achieved, and the cells were subsequently treated with graded concentrations of stimuli. sAP was assayed spectrophotometrically by using an AP-specific chromogen (which is present in the HEK detection medium supplied by the vendor) at 620 nm.

Bacterial strains and determination of MICs. *E. coli* strain 9637 and *S. aureus* strain ATCC 13709 were procured from ATCC (Manassas, VA). These two model organisms were chosen since their antimicrobial susceptibilities show a high degree of overlap with those of a panel of clinical isolates that we had examined earlier (31). The MICs of the polyamine sulfonamides were determined by the broth microdilution method, as reported previously (2). Mid-log-phase Mueller-Hinton broth (non-cation-supplemented) cultures of organisms (40 μl; the optical density at 600 nm was adjusted to 0.5 absorbance units and the broth was diluted 10-fold) were added to equal volumes of 2-fold serially diluted compounds in a 384-well microtiter plate with a Biotek Precision 2000 automated microplate pipetting system. The microtiter plates were incubated overnight at 37°C, and the absorbance at 600 nm was read. The lowest concentration that resulted in the complete inhibition of the growth of organism was recorded as the MIC.

RESULTS AND DISCUSSION

Confirmation of TLR2-dependent proinflammatory activity of LTA. LTA is a TLR2 agonist, while LPS binds to TLR4. Since we have demonstrated that several members of the acyl-polyamine class bind to and neutralize LPS (5–7, 35), it was of particular importance to examine in reporter gene assays the LTA-binding and -neutralizing activities of the polyamine sulfonamides which respond to TLR2 while obviating the false-positive and spurious results that arise due to contamination with trace quantities of LPS, which is a common problem (19). Therefore, we first verified that the LTA used was free of endotoxin by examining the NF-κB induction potency in the HEK-Blue-4 cell assay. LTA purchased from Sigma showed significant TLR4 agonistic activity (data not shown), presumably due to LPS contamination, and was therefore excluded from all further experiments. Purified LTA (from InvivoGen) isolated by the *n*-butanol procedure, however, was devoid of detectable LPS at concentrations of up to 10 μg/ml (Fig. 3A). We further verified that the TLR2-agonistic activities of LTA were not inhibited in the HEK-Blue-2 assay by polymyxin B, a specific LPS sequestrant (3, 11), even at a concentration of 2.5 mM (data not shown). It is important to note that whereas the positive control, polymyxin B, does not inhibit LTA-induced NF-κB activation in TLR2 reporter cells, the sulfonamides retain comparable activities in both systems. In the NF-κB induction assay with TLR2-expressing cells, LTA but not LPS was active (Fig. 2B). We have recently demonstrated that LTA, but not lipopeptides or peptidoglycan, induces p38 mitogen-activated protein kinase phosphorylation, CD11b expression, and cytokine and chemokine release in human blood in a manner similar to that of LPS (28). Collectively, these data confirmed that LTA is the predominant proinflammatory cytokine-inducing TLR2 agonist in human blood ex vivo and may therefore be a valid target for neutralization.

The LTA-sequestering activity, as reflected by inhibition of TLR2-mediated NF-κB induction (Fig. 4A), showed a clear dependence of the acyl chain length. The activities of both series 1 (spermine backbone) and series 2 (homosper-

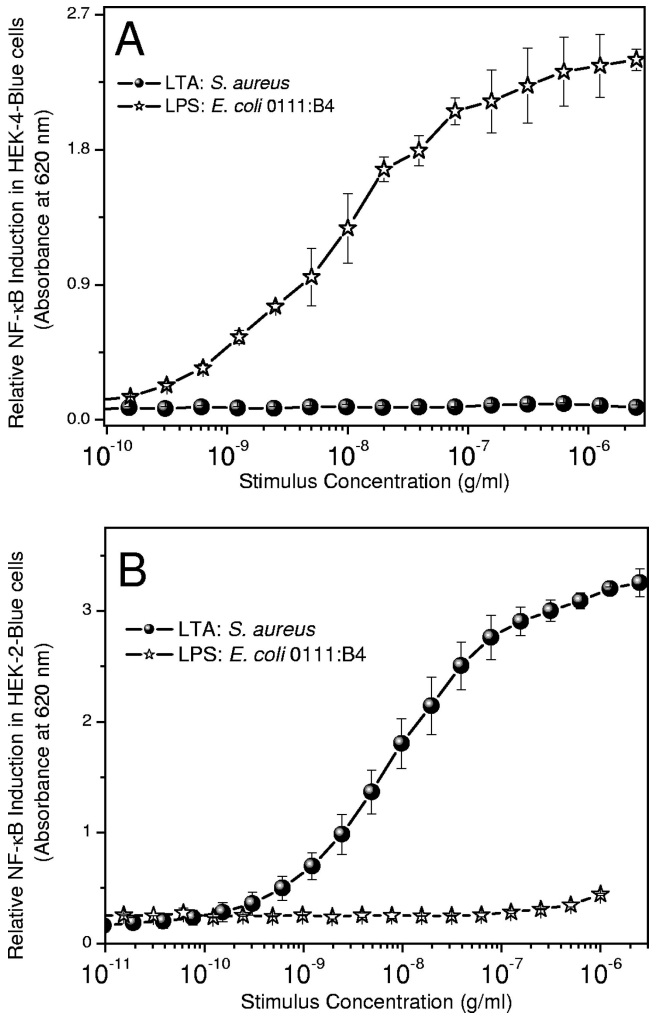


FIG. 3. (A) NF-κB induction activity of LPS and LTA in TLR4-expressing HEK-4-Blue cells treated overnight. sAP was measured colorimetrically. (B) NF-κB induction in TLR2-expressing HEK-2-Blue cells treated overnight with 1:2 serially diluted concentrations of LPS and LTA ascertained as described for panel A. Each dilution was tested in quadruplicate; coefficients of variation were less than 3%, and for clarity, only the means are shown.

mine backbone) compounds were very similar, with maximal potency (concentration inducing a half-maximal response, ~1 μM) observed for the C_{16} analogs. As has been reported earlier in the context of LPS-binding and -inhibitory activities (5), the series 3 compounds were much weaker in their LTA-neutralizing activities (Fig. 4A), presumably as a consequence of their much poorer aqueous solubility (5). A substantial correlation between LTA-sequestering potency on the one hand and LPS-neutralizing activity on the other was observed (Fig. 4B), suggesting that the interaction of these polycationic amphipathic compounds with both LTA and LPS occurs via electrostatic interactions stabilized by additional hydrophobic interactions (10, 12). Although cationic peptides have been shown to bind to and abrogate the activities of both LPS and LTA (21, 44), these results, to our knowledge, are the first describing this phenomenon in synthetic small molecules.

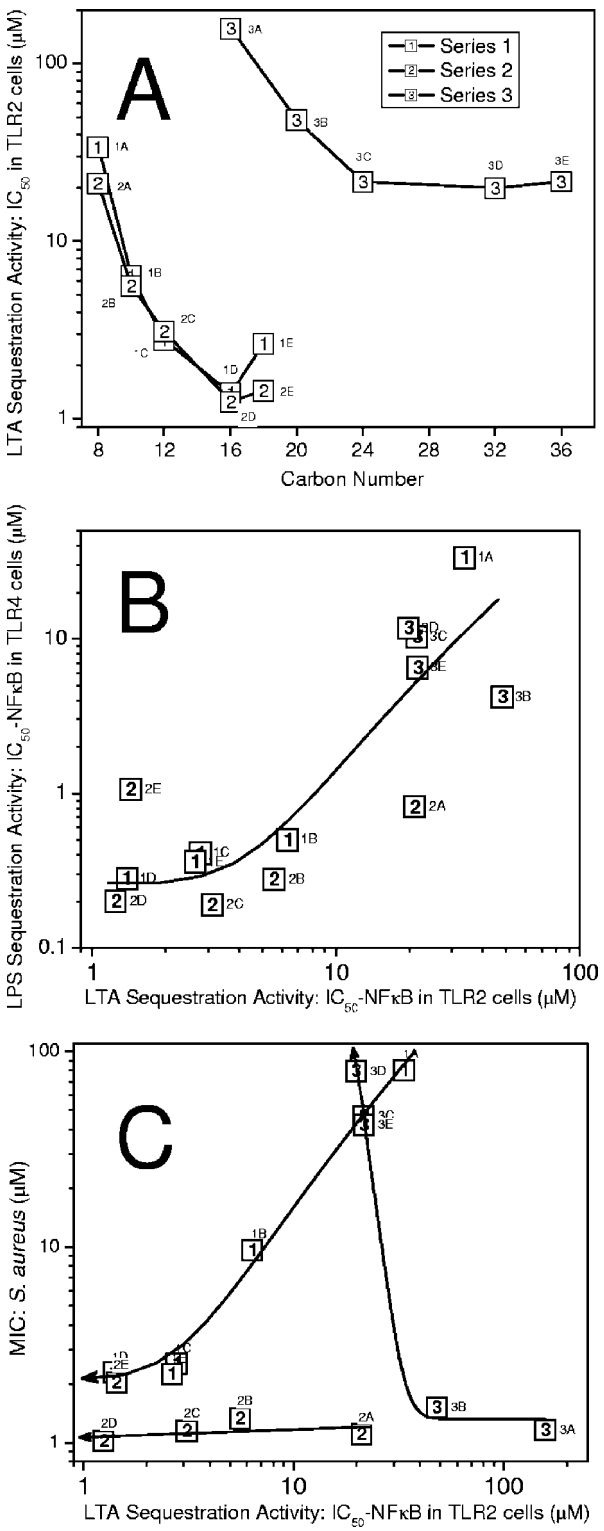


FIG. 4. (A) Correlation of carbon number of the acyl chain of the polyamine sulfonamides and their LTA-inhibitory activities, as assessed by the HEK-2-Blue NF-κB reporter gene assay. (B) Correlation of inhibition of LTA- and LPS-induced NF-κB translocation, as measured with HEK-2-Blue and HEK-4-Blue cells, respectively. Each dilution was tested in quadruplicate, and experiments were repeated three times; coefficients of variation were less than 7%, and for clarity, only the means are shown. (C) Correlation of LTA-inhibitory activity and activity against *S. aureus* strain ATCC 13709.

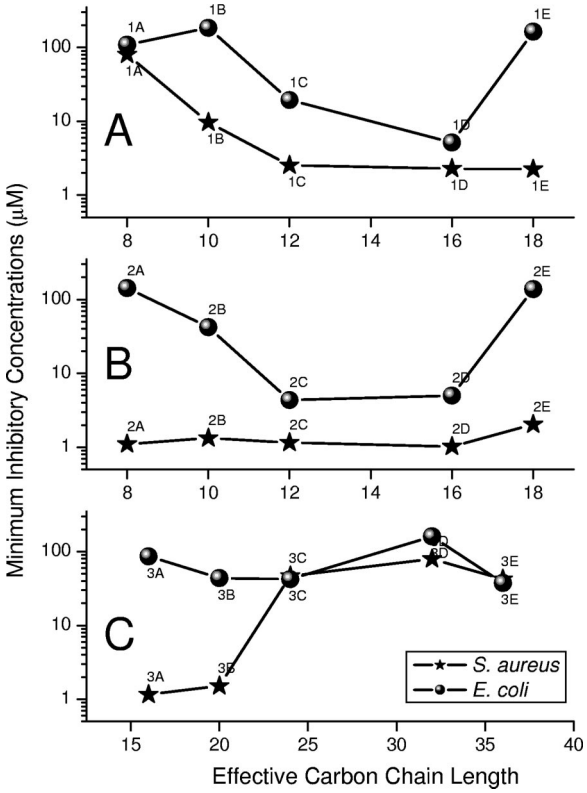


FIG. 5. MICs of series 1 (top panel), series 2 (middle panel), and series 3 (bottom panel) compounds against *E. coli* strain ATCC 9637 and *S. aureus* strain ATCC 13709 determined by the broth microdilution method. The means of triplicate data points of a representative experiment are shown.

Certain acylpolyamines have been found to significantly inhibit the growth of both gram-negative and gram-positive bacteria (2), and it is therefore not surprising that these compounds also show antimicrobial activities. We sought to examine possible correlations between perturbations of the LTA layer by using the potency of LTA sequestration as a surrogate readout and activity against a prototype gram-positive organism (*S. aureus*) with a view to identifying the equivalent of polymyxin B, a peptide antibiotic known to disrupt the supramolecular assembly of the LPS constituting the outer membranes of gram-negative bacteria (41, 47). While we found a quasilinear relationship between LTA-sequestering activity and antimicrobial activity for the series 1 compounds, all series 2 compounds were equally active against *S. aureus*, regardless of their ability to abrogate the effects of LTA. An inverse relationship was observed for the series 3 compounds (Fig. 4C). We do not yet understand the basis for these unexpected and somewhat counterintuitive findings. Our preliminary hypothesis is that either the number of protonatable charges (four in series 2 compounds and three in series 1 compounds) or the backbone itself (homospermine in series 2 compounds) is a key determinant of the activity toward the membranes of gram-positive bacteria, which appears to be relatively independent of their LTA-binding and -sequestering potencies. This conjecture is supported by our observation that the lower homologues of the series 3 bis-homospermine compounds which do show adequate aqueous solubility (compounds 3A and 3B) also exhibit potent antimicrobial activities (MIC for *S. aureus*, 1 μM) while displaying very poor LTA-sequestering activity (the concentrations inducing a half-maximal response, 60 to 110 μM). The bis-acylated series 3 analogues also bear four protonatable secondary amines with the same spacing as those of the series 1 compounds. We are examining this hypothesis by systematically expanding our screens to include compounds with scaffolds with different amine counts and spacings.

A more stringent structure-activity relationship was evident in the antimicrobial activities against *E. coli* than *S. aureus* (Fig. 5). The series 1 compounds, which had carbon chain lengths between 12 and 18, were equipotent in their MICs against *S. aureus*, whereas a distinct optimum of 16 carbons was necessary for maximal activity against *E. coli* (Fig. 5, top panel). Similarly, while all series 2 compounds were equally active against *S. aureus*, compounds 2C and 2D (12 and 16 carbons, respectively) were maximally active against *E. coli* (Fig. 5, middle panel). As mentioned earlier, compounds 3A and 3B were highly inhibitory toward *S. aureus* and were virtually inactive against *E. coli* (Fig. 5, bottom panel).

The development of resistance to membrane-active antibiotics has been relatively slower than that to conventional antibiotics, as evidenced by a resurgence in the use of polymyxin B and colistin (15, 33, 43, 48, 49), 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. The considerable in vivo data that we have obtained on related compounds that act as LPS-sequestering compounds for the prophylaxis of sepsis caused by gram-negative bacteria are indeed highly encouraging (35, 39, 45), and we are currently examining such compounds in animal models of systemic infections as stand-alone antimicrobials and as adjuncts to conventional antimicrobial chemotherapy. A more detailed understanding of the mechanisms of interactions of these compounds with LPS and LTA, as well as the gram-negative and gram-positive bacterial cell surfaces, will be instructive and should allow the rational design of analogues which combine antisepsis and antibacterial properties.

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