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Bioorganic & Medicinal Chemistry Letters 16 (2006) 1305–1308
Bioorganic & Medicinal Chemistry Letters

Novel endotoxin-sequestering compounds with terephthalaldehyde-bis-guanylhydrazone scaffolds

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Received 24 October 2005; revised 16 November 2005; accepted 17 November 2005
Available online 11 January 2006

Abstract—We have shown that lipopolyamines bind to the lipid A moiety of lipopolysaccharide, a constituent of Gram-negative bacterial membranes, and neutralize its toxicity in animal models of endotoxic shock. In an effort to identify non-polyamine scaffolds with similar endotoxin-recognizing features, we had observed an unusually high frequency of hits containing guanylhydrazone scaffolds in high-throughput screens. We now describe the syntheses and preliminary structure–activity relationships in a homologous series of bis-guanylhydrazone compounds decorated with hydrophobic functionalities. These first-generation compounds bind and neutralize lipopolysaccharide with a potency comparable to that of polymyxin B, a peptide antibiotic known to sequester LPS.

Despite tremendous advances in antimicrobial chemotherapy, the incidence and mortality due to Gram-negative sepsis continue to escalate worldwide,[1,2] and the burden on the US health care system ascribable to sepsis has been estimated at about $16.7 billion annually.[3] The primary trigger in the pathogenesis of the Gram-negative septic shock[4] syndrome is lipopolysaccharide (LPS), otherwise termed endotoxin, a constituent of the outer membrane of all Gram-negative bacteria.[5] Total synthesis of the glycolipid moiety of LPS, called lipid A (Fig. 1), has established that this is the toxic center of LPS,[6,7] and thus presents a logical therapeutic target. Indeed, Phase II clinical trials have just been successfully concluded on E5564[8–10] (Eritoran®), a tetraacyl partstructure of lipid A, which acts as a receptor antagonist of the toll-like receptor-4 (TLR-4), the principal recognition molecule for LPS in mammalian cells.[11]

Our approach has been to target circulatory LPS using small molecules.[12] The bis-anionic, amphiphilic nature of lipid A (see Fig. 1) enables it to interact with a variety of bis-cationic hydrophobic ligands, and we have found that linear bis-cationic amphipathic molecules possessing terminal, protonatable cationic groups positioned optimally (N–N distance: ~14 Å) so as to be able to simultaneously interact with the glycosidic phosphates on lipid A,[12–18] as well as appropriately positioned apolar moieties to enable hydrophobic interactions with the polyacyl domain of lipid A. Noteworthy examples of such molecules displaying potent in vitro and in vivo LPS-sequestering properties are acyl-polyamines, affording protection in animal models of sepsis.[12–14,17,18]

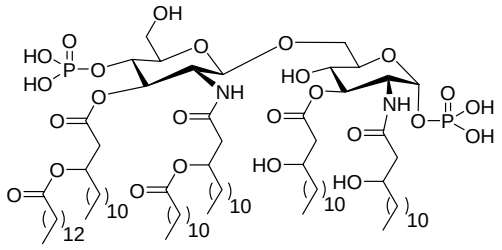

Figure 1. Structure of lipid A, the toxic center of LPS.

While members of the acylpolyamines undergo exhaustive preclinical characterization, we have been keen on exploring novel non-polyamine scaffolds and, employing an automated rapid-throughput screen[19] on focused libraries, we had recently identified several bis-guanylhydrazones as potent LPS binders.[20] However, binding to lipid A mediated via electrostatic interactions[15,16] alone

Keywords: Lipopolysaccharide; Endotoxin; Sepsis; Bis-guanylhydrazones.
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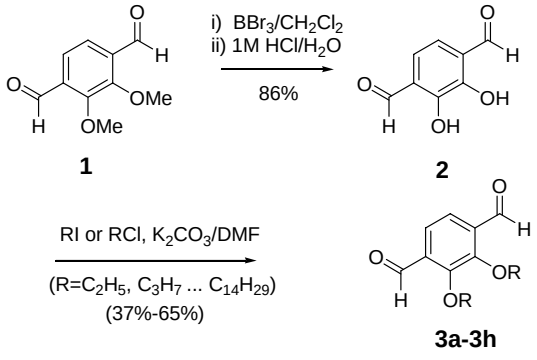
doi:10.1016/j.bmcl.2005.11.059

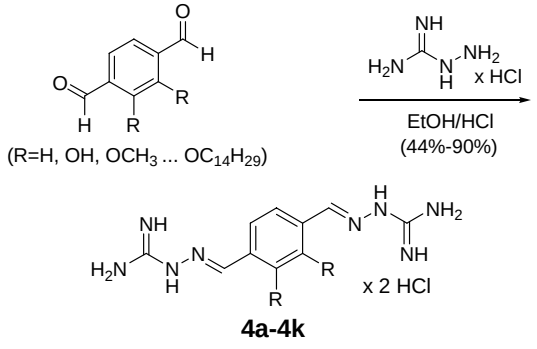
does not necessarily manifest in neutralization of endotoxicity, and we have shown that additional hydrophobic interactions with the polyacyl domain of the toxin via long-chain hydrocarbon appendages are necessary for true sequestration of the toxin.[12,18] We now describe the syntheses and preliminary structure–activity relationships in a homologous series of bis-guanylhydrazone compounds decorated with hydrophobic functionalities. These first-generation compounds bind and neutralize LPS with a potency comparable to that of polymyxin B, a peptide antibiotic known to sequester LPS.[12]

The syntheses of the target compounds are summarized in Schemes 1 and 2, and full experimental details are provided in Supplemental data. 2,3-Dimethoxyterephthalaldehyde (**1**) was prepared according to the procedure described by Kuhnert et al.[21] by double directed *ortho*-lithiation of 1,2-dimethoxybenzene with *n*-BuLi/TMEDA under reflux in ether, followed by quenching with DMF in THF. Treatment of **1** with boron tribromide in CH_2Cl_2 afforded **2** in 86% yield.[22,29] The terephthalaldehydes **3a**–**3h** were prepared by alkylation of **2** with the respective alkyl halides.[23,30] The aromatic guanylhydrazones **4a**–**4k** were obtained by condensing the terephthalaldehydes **1**–**3** with aminoguanidine HCl in refluxing ethanol/HCl (Scheme 2).[24,31] Pure aminoguanylhydrazones (as HCl salts) were isolated as crystalline products after cooling to 0 °C and washing with cold ether.

The affinities of binding of **4a**–**4k** to LPS were determined using an automated fluorescence displacement assay as described.[18,19] Inhibition of LPS-induced nitric oxide production (measured as nitrite) in murine J774 cells, and protection afforded by **4g** against lethality in a D-galactosamine-primed model of endotoxic shock in mice were performed as published previously.[17,18] The inhibition of induction of NF-κB (a key transcriptional activator of the innate immune system, leading to uncontrolled cytokine release[25,26] which ultimately leads to multiple organ failure and the shock syndrome) was quantified using human embryonic kidney 293 cells cotransfected with TLR4 (LPS receptor), CD14 and MD2 (co-receptors), available from InvivoGen, Inc. (HEK-Blue™, San Diego, CA), as per protocols provided by the vendor. Stable expression of secreted alkaline phosphatase (seAP) under the control of NF-κB/AP-1 promoters is inducible by LPS, and extracellular seAP in the supernatant is proportional to NF-κB induction. seAP was assayed spectrophotometrically using an alkaline phosphatase-specific chromogen at 620 nm using a rapid-throughput, automated protocol employing a Bio-Tek P2000 liquid handler (see Supplemental data).



Scheme 1.

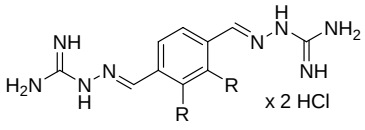


Scheme 2.

As mentioned earlier, we had observed an unexpected preponderance of guanylhydrazones compared to that of other cationic functionalities such as amines and amidines amongst hits in a high-throughput screen performed with a focused library[20] comprising of members all of which possessed the basic pharmacophore for lipid A recognition.[12,16] We surmised that the augmented binding affinity is a consequence of not only stronger salt-bridges between the ligand and the lipid A phosphate groups due to multiple H-bond donor atoms of the guanylhydrazone functionality, analogous to the 'arginine fork' hypothesis,[27] but also because of greater rigidity (compared to the highly flexible polyamines) which could account for diminished entropic loss of the free energy of binding.[16] Indeed, in silico docking of the scaffold shown in Scheme 2 to lipid A indicated excellent charge complementarity between the guanylhydrazone moieties and lipid A phosphates, with the distance between the terminal charged groups being 14.14 Å, commensurate with the inter-phosphate distance in lipid A[12] (data not shown). We sought to examine if the introduction of additional alkyl functionalities would confer true LPS-neutralizing activity and to determine the optimal chain length of the alkyl group corresponding to maximal biological potency. The concentrations of the bis-guanylhydrazones corresponding to effective displacement of 50% of the bound fluorescent probe (ED_{50}; relative affinity of binding) and of the inhibition (IC_{50}) of nitric oxide (NO) and NF-κB are summarized in Table 1.

As shown in Figure 2, dependencies on alkyl chain length are observed for binding affinity, as well as NO and NF-κB neutralization. For binding affinity, the optimal substituent appears to be C_4H_9, while in both in vitro bioassays, the most favorable hydrophobic group is C_8H_{17}. This discrepancy is due to the fact that in the homogeneous fluorescent displacement assay[19] performed in aqueous buffer, the ligand must be completely soluble; increasing hydrocarbon chain length substituents results in a progressively retarded aqueous

Table 1. Summary of binding affinity and biological activity for terephthalaldehyde-bis-guanylhydrazones **4a–4k**



Compound	R	ED_{50} (μM)	IC_{50} (μM)	
			NO	NF-κB
Polymyxin B (Ctrl)		1.67	0.980	1.23
4a	–H	34.6	24.4	164
4b	–OH	43.7	26.4	59.1
4c	$–OCH_3$	42.3	28.0	175
4d	$–OC_2H_5$	22.8	4.56	35.6
4e	$–OC_3H_7$	12.7	8.38	27.6
4f	–O-Allyl	29.9	9.64	61.4
4g	$–OC_4H_9$	9.44	4.15	9.40
4h	$–OC_5H_{11}$	7.82	3.54	10.5
4i	$–OC_6H_{13}$	13.2	4.67	6.11
4j	$–OC_8H_{17}$	39.5	3.45	4.50
4k	$–OC_{14}H_{29}$	>500	14.9	190

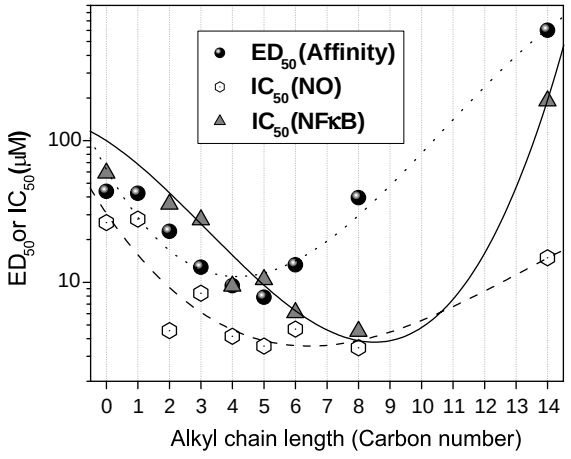


Figure 2. Relationship of alkyl chain length of **4a–4k** with binding affinity and in vitro LPS neutralization potency.

solubility, resulting in higher ED_{50} values. In the NO and NF-κB neutralization assays, the presence of fetal bovine serum in the cell-culture medium ensures complete and uniform solubility, and obviates this problem.[14,18] It may also be pointed out that the NF-κB IC_{50} values best correlate with inhibition of cytokine release ex vivo in human whole blood experiments which, in turn, correspond best to in vivo protection in animal models (unpublished data).

Given that **4j** was the most potent compound in this assay (Table 1), we elected to characterize this compound in an in vivo D-galactosamine-primed mouse model of endotoxic shock. It is worth noting that the cytokine response to LPS in mice closely parallels that of humans[28] and is thus a well-established confirmatory assay.[17,18] The administration of graded doses of **4j** resulted in a dose-dependent decrease in lethality in mice challenged with supralethal (200 ng/mouse; LD_{100}: 100 ng/mouse) doses of LPS (Fig. 3).

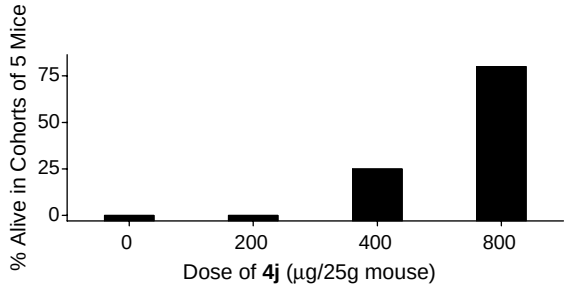


Figure 3. Dose-dependent protective effect of **4j** in a murine model of lethality induced by 200 ng LPS.

These results lend support to our strategy of incrementally converting high-affinity LPS binders to true LPS sequestrants by appending suitable hydrophobic functionalities. Further, these data would suggest that a systematic exploration of the bis-guanylhydrazone scaffold may yield useful anti-endotoxin molecules. It is gratifying that these first-generation compounds should display significant in vivo activity, given that the placement of the hydrophobic groups in the **4** series is not optimal, for we have shown earlier[12–16,18] that as a consequence of favorable steric properties in the ligand:lipid A complex, substitution with long-chain hydrocarbon groups at the ends of the ligand, rather than at the center, corresponds to a significantly higher biological activity. We are presently planning the synthesis and evaluation of such compounds.

Acknowledgments

This work was supported by NIH 1U01 AI 56476. K.K. is grateful to the Royal Thai Government for a scholarship from the Development and Promotion of Science and Technology (DPST) Project. Coordinates of models of the compounds docked on lipid A are available on request.

Supplementary data

Supplementary data and can be found, in the online version, at doi:10.1016/j.bmcl.2005.11.059.

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29. 2,3-Dihydroxyterephthalaldehyde (**2**).[22] To a solution of **1** (400 mg, 2 mmol) in anhydrous CH_2Cl_2 (1 mL) at rt was added dropwise a 1.0 M boron tribromide solution in anhydrous CH_2Cl_2 (20 mL, 20 mmol). The reaction mixture was stirred for 1 h, followed by quenching of the reaction by addition of 1 M of aq HCl (20 mL). The mixture was extracted with CH_2Cl_2 (3× 100 mL). The combined organic extracts were washed with brine (2× 50 mL) and dried over Na_2SO_4 and the solvent was removed under reduced pressure. The resulting viscous liquid was purified by flash column chromatography (1% MeOH, 1% AcOH and CH_2Cl_2) on silica gel to obtain compound **2** (239 mg, 86%) as a yellowish powder.
30. General procedure for the synthesis of **3a**–**3h**.[23] To a solution of **2** (10 mg, 0.06 mmol) in anhydrous dimethylformamide (4 mL) were added the respective alkyl halides (25 equiv) [C_2H_5I, C_3H_7I, C_3H_5I, C_4H_9Cl, $C_5H_{11}I$, $C_6H_{13}Cl$, $C_8H_{17}Cl$, and $C_{14}H_{29}Cl$] and K_2CO_3 (67 mg, 0.48 mmol). The reaction mixture was stirred for 15 h at rt. The reaction was quenched with water (5 mL), and the resulting solution was extracted with CH_2Cl_2, and the combined organic extracts were washed with brine and dried over Na_2SO_4. The resulting viscous liquids were purified by flash column chromatography (CH_2Cl_2/hexane = 6:4) to obtain the terephthalaldehyde analogs **3a**–**3h** (**3a**, R=C_2H_5; **3b**, R=C_3H_7; **3c**, R = allyl; **3d**, R=C_4H_9; **3e**, R=C_5H_{11}; **3f**, R=C_6H_{13}; **3g**, R=C_8H_{17}; **3h**, R=$C_{14}H_{29}$) as oils.
31. General procedure for the synthesis of **4a**–**4k**.[24] To a hot solution of aminoguanidine hydrochloride (13.7 mg, 0.12 mmol) in EtOH (1 mL) was added a solution of the respective terephthalaldehyde (**1**, **2**, and **3a**–**3h**, 0.5 equiv, in 0.5 mL EtOH) and concd HCl (0.1 mL). The reaction mixture was heated up to 80 °C for 1 h, and then cooled to 0 °C. The resultant precipitate was filtered and washed with cold ether (2 mL) to give bis-guanylhydrazone analogs **4a**–**4k**. All ^1H and ^{13}C spectra, and mass spectral data were consonant with their corresponding structures.