Pergamon

Bioorganic & Medicinal Chemistry 10 (2002) 47–55

BIOORGANIC & MEDICINAL CHEMISTRY

In Vitro and In Vivo Inhibition of LPS-Induced Tumor Necrosis Factor-α Production by Dimeric Gallotannin Analogues

Ken S. Feldman,[a,*] Sarah L. Wilson,[a] Michael D. Lawlor,[a] Charles H. Lang[b] and William J. Scheuchenzuber[c]

[a]*Department of Chemistry, The Pennsylvania State University, University Park, PA 16802, USA*
[b]*Department of Cellular and Molecular Physiology, The Pennsylvania State University College of Medicine, Hershey, PA 17033, USA*
[c]*Life Sciences Consortium, The Pennsylvania State University, University Park, PA 16802, USA*

Received 4 May 2001; accepted 29 June 2001

Abstract—Designed dimeric gallotannin analogues featuring two tetragalloylglucopyranose cores connected by various hydrocarbon linkers inhibit tumor necrosis factor-α secretion from lipopolysaccharide-stimulated human peripheral blood mononuclear cells by up to 53% (5–24 μM concentration range) compared to control. Comparable suppression of tumor necrosis factor-α levels (~50% vs control) was observed in the plasma of rats co-treated with lipopolysaccharide and specific tannin analogues selected for their lack of interleukin 1-β stimulating activity.

Introduction

The secretion of cytokines from activated macrophages and related cells is an integral component of an effective immune response to a viral or bacterial infection. However, many autoimmune and inflammatory diseases are linked in part to chronic overproduction of the cytokine tumor necrosis factor-α (TNF-α).[1] In addition, a surge of proinflammatory cytokines such as TNF-α[2,3] and interleukin 1-β (IL-1β) as a result of exposure to Gram-negative bacterial lipopolysaccharide (LPS, active component = lipid A) can lead to life-threatening septic shock. Consequently, therapeutic strategies targeted toward diminishing plasma TNF-α levels have received much attention. Protocols designed to achieve this goal have run the gamut from blocking the initial lipid A–receptor(s) interaction[4,5] as an anti-sepsis therapy to sequestering nascent serum TNF-α with antibodies and soluble receptors.[6,7] In between these extreme points of intervention, small molecules such as thalidomide derivatives[8,9] and nucleoside analogues[10] have displayed promise as inhibitors of enzymes or receptors in the macrophage cytosolic signaling pathway, while peptide-based metalloproteinase inhibitors, which may prevent cleavage of the first-formed pro-TNF-α protein to its soluble form, are under investigation.[6] Promising results have been reported upon application of some of these strategies to the treatment of certain inflammatory diseases, but attempts to ameliorate the devastating effects of septic shock have met with little success.

The naturally occurring gallotannin β-pentagalloylglucopyranose [β-PGG, (**1**), Fig. 1], recently has been identified as an effective inhibitor of LPS-induced TNF-α release both from human peripheral blood mononuclear cells (h-PBMCs) and in live rats.[11] The polyphenolic tannins do not structurally or functionally resemble any of the therapeutic agents discussed above, and so this result draws attention to the possibility that prospecting among members of the tannin class of secondary plant metabolites[12–14] may generate novel leads against septic shock and other inflammatory diseases. β-PGG reduced the output of TNF-α in vitro by as much as 90% versus control. However, in vivo studies with rats exposed severe limitations in using this simple monomeric tannin as a treatment for septic shock. Despite lowering plasma TNF-α levels in LPS-treated rats versus control, the physiological symptoms of septic shock (increased heart rate and blood glucose levels, decreased blood pressure) were not measurably dampened. It is plausible that the sustained septic shock symptoms can be traced to the β-PGG-stimulated secretion of the companion cytokine

*Corresponding author. Tel.: +1-814-864-4654; fax: +1-814-863-8403; e-mail: ksf@chem.psu.edu

0968-0896/02/$ - see front matter
PII: S0968-0896(01)00251-6

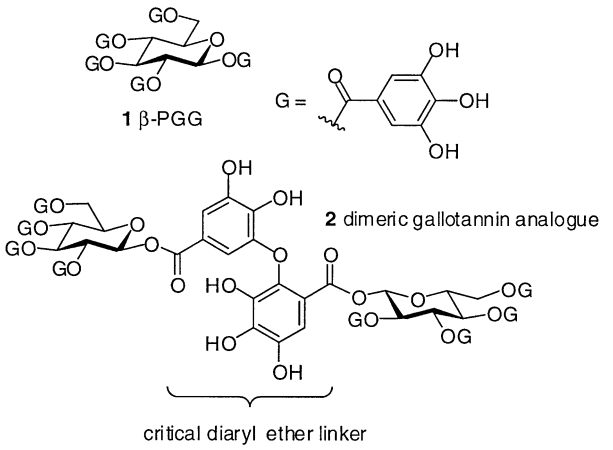


Figure 1. Immunomodulatory tannins, β-D-pentagalloylglucopyranose (**1**), and a dimeric tannin analogue **2**.

IL-1β,[11] a known mediator of this response.[15] Thus, the design, synthesis, and evaluation of second-generation tannin-based immunomodulators which did not themselves generate IL-1β became a high priority.

A design hypothesis emerged from the observation that the dimeric gallotannin analogue **2**, as well as the structurally related naturally occurring dimeric ellagitannins coriariin A and agrimoniin, stimulated secretion of TNF-α from h-PBMCs, whereas the monomeric species **1** was largely inert at similar concentrations.[16] Furthermore, the dose-response data for the dimeric species were reminiscent of that recorded after exposure of h-PBMCs to LPS. Taken together, these results might be accommodated by a mechanism-of-action model which features interaction of the dimeric tannins with the obligate PBMC receptors, surface bound CD14 and soluble LBP,[17] that mediate the LPS-initiated septic shock response. Although the details of the lipid A–receptor interaction are far from secure, some data are consistent with a model in which an appropriate three-component interaction between the two receptors and lipid A is necessary to trigger the signaling cascade that begins by engaging another cell surface protein, TLR4.[18] It is not beyond the realm of speculation, therefore, to posit that the active dimeric tannins might present galloylated glucopyranose recognition elements to each of the receptors, and that the digalloyl ether linker common to all of these active compounds (cf. **2**) might be a critical element in orienting these putative binding domains in a manner that facilitates productive interaction between the bound receptors and TLR4. Circumstantial evidence (vide infra) is suggestive of no more than a secondary role for the diaryl ether linker in the recognition event(s). In this scenario, the monomeric species **1** may bind to one or both of the receptors in competition with lipid A, but would provide no organizing framework to the receptor complex, and hence fail to initiate signal transduction. This working hypothesis can be tested by assembly of dimeric tannin constructs that preserve the tetragalloylglucopyranose endcaps but alter their relative orientation through incorporation of linkers that differ from the digalloyl ether moiety in **2**. In the best case, members of this class of molecules should be effective antagonists of LPS. In addition, it is possible that a larger and more complex tannin architecture might benefit from increased selectivity in protein interactions[19] and therefore require smaller doses with potentially fewer unwanted side effects than those observed with the monomer **1**. This model does not permit predictions about IL-1β stimulating activity, and so this point will have to be determined through experiment.

Results and Discussion

Synthesis chemistry

The general strategy for preparation of the dimeric tannins involved the coupling of selected diacid chlorides with 2 equiv of per-*O*-benzyl tetragalloylglucopyranose (**7**).[20] Stereochemically pure compounds were desired to limit the number of structural variables in interpreting the immunological assays, and this coupling procedure is reported to provide isomerically pure β-anomeric ester linkages using aromatic monoacid chlorides.[20,21] Five compounds **3a–e** were selected for initial evaluation (Fig. 2). Dimer **3a**, the most structurally conservative change, probes the effect of hydroxylation on the presumably critical diaryl ether linker of the active compounds. The alkyl linker in **3b** provides more flexibility and more hydrophobicity than the conformationally limited diaryl ether of **2**. Compounds **3c–e** are all more rigid than the active diaryl ether-bearing compounds, and the different aryl spacers enforce different core–core distances and orientations compared with **2**.

The requisite acid chlorides **6b–d** are commercially available, and biphenyl diacid chloride **6e**[22] was readily prepared from the corresponding diacid. Diaryl ether diacid chloride **6a** was prepared in two steps from the known Ullmann coupling product **4** (Scheme 1).[23] Galloylated glucopyranose **7** was coupled with each of

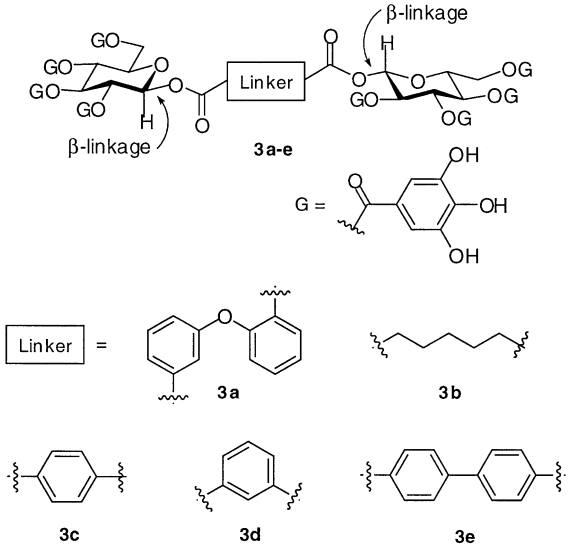
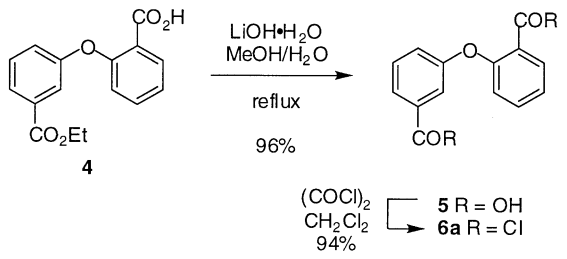


Figure 2. Five dimeric tannin analogues designed as LPS antagonists in a septic shock response.

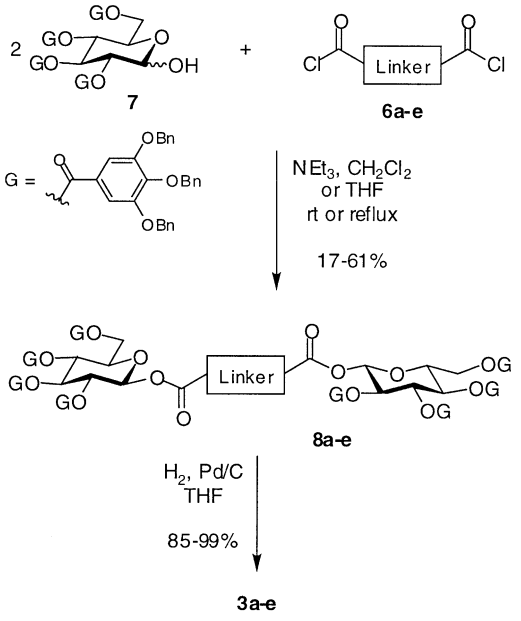


Scheme 1.

the diacid chlorides **6a–e** using triethylamine as base in CH_2Cl_2 to furnish dimers **8a–e** in isolated yields of 17–61% (Scheme 2). These successful bis acylations stand in sharp contrast to previous attempts to prepare dimer **2** using a similar procedure, suggesting that the bis acid chloride corresponding to the 2,3,4,4′5′-penta-*O*-benzyl version of **6a** is an uncharacteristically poor acylating reagent.[20] As anticipated, compounds **8a**, **8c**, and **8d** were formed with good diastereoselectivity for the β,β′-anomer as determined by ^{1}H NMR. Higher temperature (refluxing THF) was necessary to prepare the biphenyl-linked dimer **8e**, which was obtained as a 4:1 ratio of β,β′-to-α,β′ anomers. The coupling reaction to form **8b** resulted in a mixture of the three possible anomeric isomers, but the β,β′ anomer was produced as the major product, and α,α′ diester formation was completely suppressed by the slow addition of diacid chloride **6b** to alcohol **7**.[21] Debenzylation of the phenolic hydroxyl protecting groups in dimers **8a–e** proceeded in excellent yield to furnish the desired tannin analogues **3a–e**.

In vitro immunomodulatory activity

The five dimers were initially tested for their ability to induce TNF-α secretion from h-PBMCs. The cells were isolated and incubated with each of the dimeric gallotannins **3a–e** for 24 h, and the amount of TNF-α in the culture supernatants was determined by ELISA (Fig. 3).[16] In each case, LPS was used as a positive control while untreated cells were reserved as negative controls. Two of the compounds, **3b** and **3d**, showed very low levels of TNF-α production, and **3c** and **3e** produced no measurable response from the h-PBMCs (data not shown). Treatment of the h-PBMCs with the diaryl ether-containing compound **3a**, however, a species which retains much the same overall shape as the active dimeric gallotannin **2**, led to significant cytokine release. Despite lacking the active compounds' diaryl ether hydroxyls, **3a** appears to act as an immunostimulator and was therefore eliminated from further consideration as a TNF-α inhibitor. This result also seems to lessen the likelihood that the linker unit itself is a critical recognition element for association with the receptors, in accord with an implicit assumption of the design hypothesis.



Scheme 2.

Compounds **3b–e** were then evaluated as mediators of IL-1β production from h-PBMCs (Fig. 4). This cytokine is also released as part of a septic shock response to bacterial LPS exposure, and so dimers that caused minimal IL-1β release would hold the greatest therapeutic promise. The *meta*-phenyl dimer **3d** led to significant IL-1β production at higher concentrations (30 μM, 2110 pg/mL), while the *para*-phenyl isomer **3c** displayed significantly less activity (30 μM, 690 pg/mL). The other two substrates, **3b** and **3e**, ostensibly failed to induce IL-1β secretion from the cells (<250 pg/mL at 30 μM), thus emerging as the top inhibitory candidates for further in vitro and in vivo study.

TNF-α inhibition data for compounds **3b–e** are shown in Figures 5 and 6. h-PBMCs were incubated with a fixed concentration of LPS (5 μg/mL for **3b–c**, 10 μg/mL for **3d–e**) for 45 min, followed by addition of various concentrations of each dimeric tannin. The cell

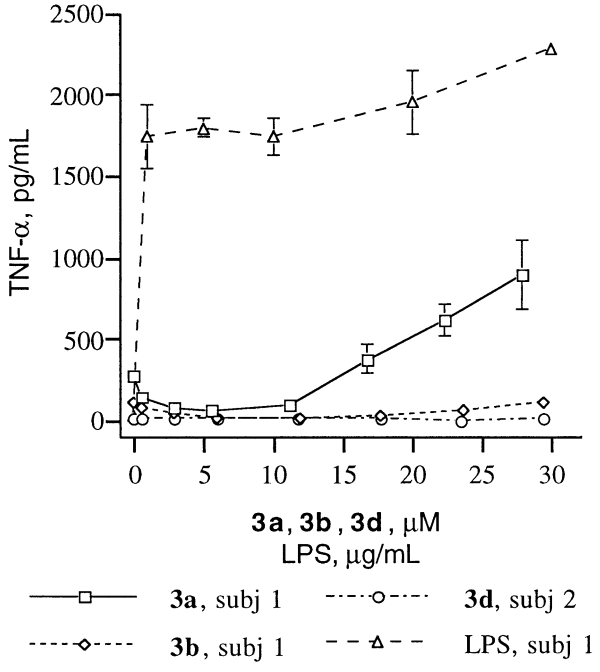


Figure 3. TNF-α secretion from two subjects' PBMCs upon exposure to varying concentrations of dimeric tannins **3a,b, d** and LPS (24 h).

culture supernatants were harvested after 8 h^{24} and assayed by ELISA for TNF-α release. To compensate for the inherent variability of using cells from different subjects, the cytokine secretion data were normalized by calculating a percent of the control value, represented by LPS treatment only.

All of the dimers inhibited TNF-α production in the concentration range studied, 0–24 μM. The pentyl and *para*-phenyl-linked dimers, **3b** and **3c**, respectively, exhibited promising activity with maximum inhibition values of 30% for both **3b** and **3c** at 5 μM at 23.5 μM for **3b** (Fig. 5). The most potent inhibitor of TNF-α secretion was the *meta*-phenyl-linked dimer **3d**, with maximum inhibition of 53% at 11.8 μM (Fig. 6). The biphenyl-linked compound **3e** was slightly less potent at 27%. However, in a separate trial using a dose of 1 μg/mL of LPS as an h-PBMC stimulant, a higher maximum inhibition value of 45% was observed with **3e** at a concentration of 5.6 μM. Eliminating LPS pre-treatment by introducing the compounds and LPS at the same time had little effect on the maximum inhibitory values (data not shown).

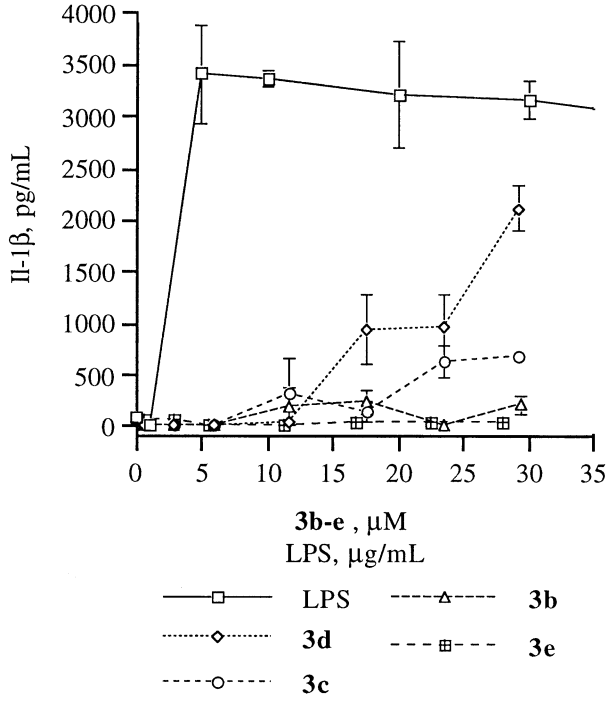


Figure 4. IL-1β secretion from subject 3′s PBMCs upon exposure to varying concentrations of dimeric tannins **3b–e** and LPS (24 h).

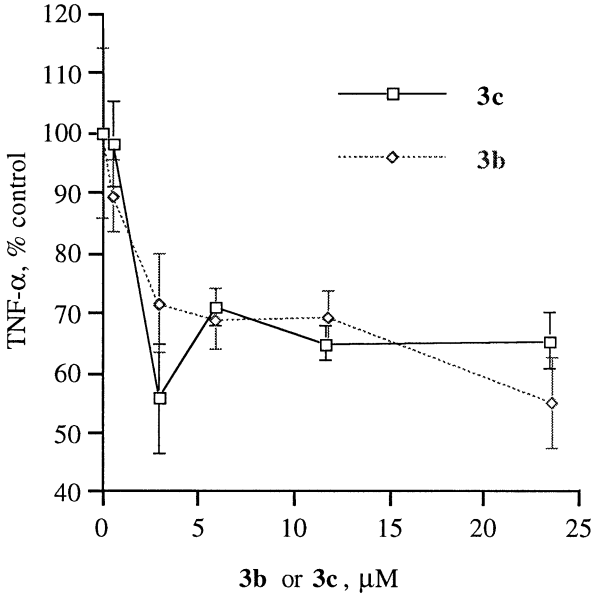


Figure 5. TNF-α secretion from subject 4′s PBMCs upon exposure to 5 μg/mL of LPS and varying concentrations of **3b** or **3c** (8 h).

In vivo immunomodulatory activity

Compounds **3b** and **3e** were chosen for study in a rat model system since they appear incapable of inducing secretion of significant quantities of IL-1β from h-PBMCs and are therefore less likely to initiate or sustain a septic shock response. The experiment involved nine animals: rats 1–4 were administered LPS (1 mg/kg) and one of the two dimeric tannins (20 mg/rat over 180 min), whereas rats 5–8 were controls and received only the initial LPS treatment, and rat 9 received saline only (data not shown). Plasma samples were collected from each animal at 90 and 180 min for determination of cytokine levels by ELISA-based analysis. The TNF-α data (Fig. 7) indicated significant reduction ($\leq$50%) of cytokine levels at 90 min in three of the four tannin-treated rats, with an average decrease of 58%. Encouragingly, the rats dosed with **3b** and **3e** did not produce higher IL-1β amounts than the LPS controls at 180 min (Fig. 8), and, in fact, the cytokine levels are 17% lower on average in these rats. Unfortunately, monitoring typical physiological symptoms of

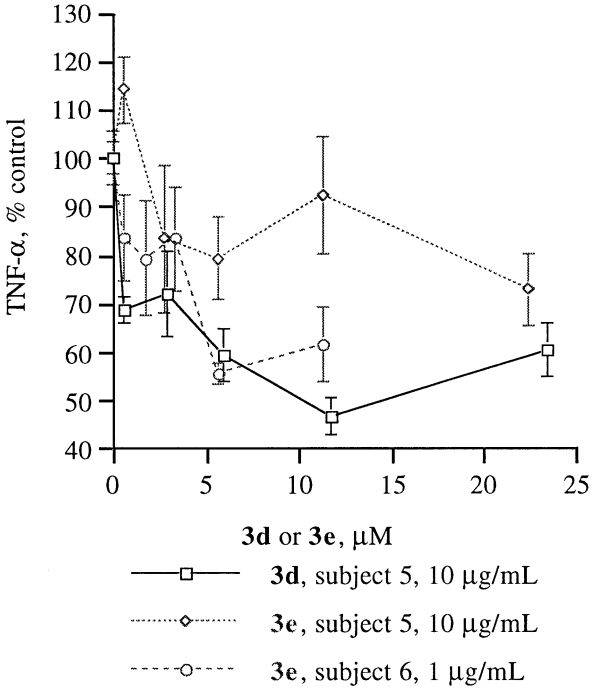


Figure 6. TNF-α secretion from subject 5's and subject 6's PBMCs upon exposure to 1 or 10 μg/mL of LPS and varying concentrations of **3d** or **3e** (8 h).

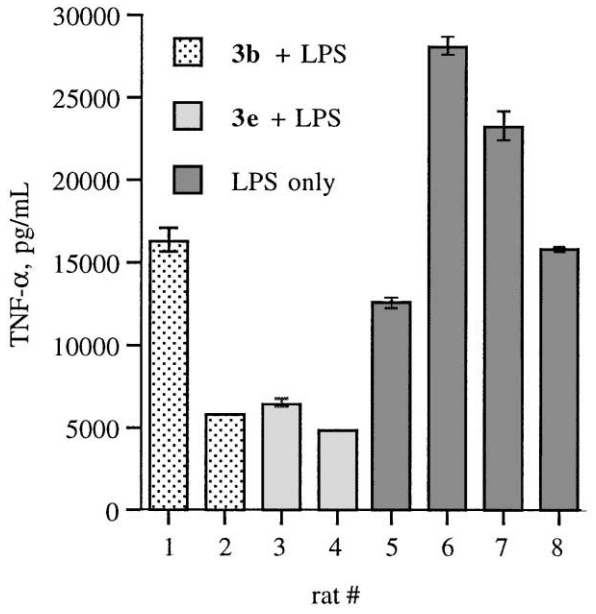


Figure 7. Plasma TNF-α levels in rats at 90 min after treatment with LPS (1 mg/kg) and **3b** (12.5 mg), or LPS and **3e** (12.5 mg), or LPS only (1 mg/kg).

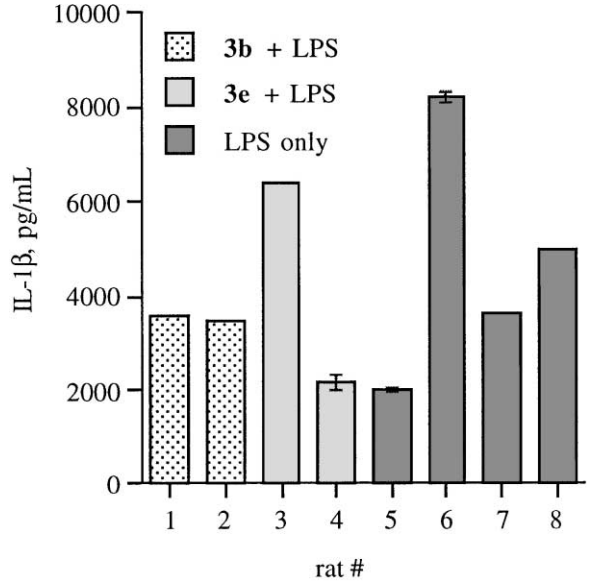


Figure 8. Plasma IL-1β levels at 180 min from rats treated with LPS (1 mg/kg) and **3b** (20 mg), or LPS and **3e** (20 mg), or LPS only (1 mg/kg).

septic shock in the rats so treated (arterial blood pressure, heart rate, plasma glucose level) did not reveal any broadly significant amelioration of the LPS-induced effects. Heart rate, however, did return to pre-LPS levels upon treatment with **3e** (average beats/min at 180 min: after LPS administration, 483 ± 10; after LPS + **3e**, 385 ± 33; saline control, 380 ± 22).

Conclusions

Building upon earlier results in which the simple gallotannin **1** was identified as a promising down-regulator of LPS-mediated TNF-α secretion in both h-PBMCs and in live rats, five dimeric gallotannin analogues were prepared and analyzed for immunomodulatory activity. Four of these compounds displayed significant inhibitory properties in vitro, demonstrating that a great deal of structural variability is tolerated in the linker spanning the two glucopyranose cores. While not as potent in vitro as β-PGG, synthetic dimers **3b** and **3e** showed similar activity in vivo without exacerbating the LPS-initiated septic shock response as observed with monomer **1**. The mechanism by which tannins operate as immunomodulators is a matter of speculation at present and will be the subject of continuing work in this area.

Experimental

General

All reactions were performed, with magnetic stirring, in flame-dried glassware under a positive pressure of argon. Commercial grade reagents and solvents were used without further purification except as indicated below. Dichloromethane, benzene, and triethylamine were distilled from calcium hydride. THF was distilled from sodium benzophenone ketyl or dianion. Methanol was distilled from magnesium. *N*,*N*-Dimethylformamide (DMF) was sequentially dried in three stages over activated 3 Å molecular sieves. Reaction product solutions and chromatography fractions were concentrated using a Büchi rotary evaporator at approximately 20 mmHg and then at ca. 0.1 mmHg (vacuum pump). Column chromatography was performed using 32–63 μm silica gel and the indicated solvent. Infra-red (IR) spectra were recorded on a Perkin–Elmer 1600 Series FT infrared spectrophotometer. Magnetic resonance spectra (^{1}H NMR, ^{13}C NMR) were recorded on either a Bruker DPX-300, AMX-360, or DRX-400 spectrometer. Chemical shifts are reported in δ units using tetramethylsilane (TMS) or acetone as an internal standard for ^{1}H NMR and chloroform or acetone as the internal standard for ^{13}C NMR. High resolution fast atom bombardment (FAB) mass spectra were run at the University of Texas at Austin, TX, USA. Combustion analyses were performed by Midwest Microlabs, Indianapolis, IN, USA.

General procedure A: bisacylation of 2,3,4,6-tetrakis(3,4,5-tribenzyloxybenzoyl)-D-glucose (7). To a solution of 2,3,4,6-tetrakis(3,4,5-tribenzyloxybenzoyl)-D-glucose (**7**)[20] (300 mg, 0.160 mmol) in 1.5 mL of CH_2Cl_2 was added triethylamine (67 μL, 0.48 mmol) followed by the appropriate diacid chloride (0.5 equiv). In some cases (see text), slow addition of a solution of the diacid chloride in CH_2Cl_2 dropwise via syringe pump over ca. 6 h afforded increased selectivity for the β-anomeric linkage in the products. This solution was stirred at rt for 18 h. The reaction mixture was treated with 1 N HCl and extracted with EtOAc. The organic layer was washed sequentially with H_2O and brine, and then dried over Na_2SO_4. After filtration and removal of the solvents in vacuo, the product was purified by flash chromatography using 3:5:12 EtOAc/benzene/hexane.

General procedure B: hydrogenation. To a solution of the appropriate benzylated dimer in THF was added 10% Pd/C (0.5–1 equiv Pd). The reaction mixture was purged four times with H_2 and stirred at rt under one atm of H_2 for 18 h. The reaction mixture was filtered through Celite which was rinsed with acetone. After concentration, the resulting gray solid was triturated with hexane and ether and dried under vacuum at 35 °C.

1,1′-*O*-2,2′,3,3′,4,4′,6,6′-Tetrakis(3,4,5-tribenzyloxybenzoyl)-β,β′-D,D′-glucopyranosylterephthalate (8c). By use of general procedure A, terephthaloyl chloride (16 mg, 0.079 mmol) was coupled alcohol **7** and purified by flash chromatography to afford 190 mg (61%) of benzylated dimer **8c**: IR ($CDCl_3$) 1734 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 8.02 (s, 4H), 7.43–7.15 (m, 136H), 6.25 (d, $J=8.3$ Hz, 2H), 6.05 (appar t, $J=9.8$ Hz, 2H), 5.82 (dd, $J=10.2$, 8.3 Hz, 2H), 5.70 (appar t, $J=9.8$ Hz, 2H), 5.11–4.91 (m, 48H), 4.77 (d, $J=9.4$ Hz, 2H), 4.47–4.42 (m, 2H), 4.32 (dd, $J=11.9$, 6.2 Hz, 2H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 165.6, 165.5, 165.0, 164.8, 163.7, 152.6, 152.5, 152.4, 143.2, 143.1, 142.6, 137.4, 137.3, 137.2, 136.6, 136.3, 136.2, 132.9, 130.3, 128.5, 128.5, 128.4, 128.2, 128.2, 128.1, 128.0, 127.8, 127.5, 124.4, 123.6, 123.5, 109.3, 109.1, 109.0, 93.2, 75.1, 73.4, 73.3, 71.1, 71.1, 71.0, 69.8, 63.19; MS (+FAB) 3867 (MH^+, 16). Anal. calcd for $C_{244}H_{202}O_{46}$: C, 75.74, H, 5.23. Found: C, 75.59, H, 5.29.

1,1′-*O*-2,2′,3,3′,4,4′,6,6′-Tetrakis(3,4,5-trihyroxybenzoyl)-β,β′-D,D′-glucopyranosylterephthalate (3c). By use of general procedure B, dimer **8c** (150 mg, 0.039 mmol) was hydrogenated using 32 mg (0.03 mmol) of Pd/C in 5 mL of THF to afford 30 mg (99%) *para*-phenyl linked dimer **3c**: IR (KBr) 3422, 1702 cm^{-1}; ^1H NMR ($(CD_3)_2CO$, 300 MHz) δ 8.5-7.4 (m, 24H), 8.05 (s, 4H), 7.13 (s, 4H), 7.02 (s, 4H), 6.97 (s, 4H), 6.94 (s, 4H), 6.37 (d, $J=8.3$ Hz, 2H), 6.02 (appar t, $J=9.6$ Hz, 2H), 5.65 (appar t, $J=9.8$ Hz, 2H), 5.64–5.58 (m, 2H), 4.61–4.54 (m, 4H) 4.38 (dd, $J=12.6$, 4.3 Hz, 2H); ^{13}C NMR [$(CD_3)_2CO$, 75 MHz] δ 165.5, 164.9, 164.9, 164.7, 163.3, 145.1, 145.1, 145.0, 138.5, 138.3, 138.2, 135.9, 133.3, 130.0, 128.4, 120.5, 119.7, 119.6, 119.4, 109.4, 109.2, 93.2, 73.2, 72.1, 70.9, 68.3, 61.8; MS (+FAB) 1706 (MH^+ 7); HRFABMS calcd for $C_{76}H_{58}O_{46}$: 1706.2199. Found: 1706.2232.

1,1′-*O*-2,2′,3,3′,4,4′,6,6′-Tetrakis(3,4,5-tribenzyloxybenzoyl)-β,β-D-glucopyranosylisophthalate (8d). By use of general procedure A, isophthaloyl chloride (16 mg, 0.079 mmol) was coupled to alcohol **7** and purified by flash chromatography to afford 150 mg (48%) of benzylated dimer **8d**: IR ($CDCl_3$) 1730 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 8.76 (s, 1H), 8.19 (dd, $J=7.9$, 1.5 Hz, 2H) 7.42–7.15 (m, 137H), 6.26 (d, $J=8.2$ Hz, 2H), 6.01 (appar t, $J=9.4$ Hz, 2H), 5.81 (dd, $J=9.8$, 8.3 Hz, 2H), 5.74 (appar t, $J=9.6$ Hz, 2H), 5.09–4.90 (m, 48H), 4.76 (d, $J=9.0$ Hz, 2H), 4.41–4.30 (m, 4H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 165.7, 165.5, 165.0, 164.9, 163.6, 152.6, 152.6, 152.4, 143.2, 143.1, 142.6, 137.5, 137.3, 137.3, 136.6, 136.4, 136.4, 136.3, 129.3, 128.9, 128.9, 128.7, 128.5, 128.4, 128.3, 128.1, 128.0, 127.8, 127.7, 127.6, 124.4, 123.7, 123.6, 109.3, 109.1, 93.1, 75.2, 75.1, 73.3, 73.2, 71.2, 71.2, 71.0, 69.7, 63.1; MS (+FAB) 3867 (MH^+, 7). Anal. calcd for $C_{244}H_{202}O_{46}$: C, 75.74, H, 5.23. Found: C, 75.61, H, 5.23.

1,1′-*O*-2,2′,3,3′,4,4′,6,6′-Tetrakis(3,4,5-trihydroxybenzoyl)-β,β′-D-glucopyranosylisophthalate (3d). By use of general procedure B, dimer **8d** (150 mg, 0.039 mmol) was hydrogenated using 32 mg (0.03 mmol) of Pd/C in 5 mL of THF to afford 50 mg (76%) of *meta*-phenyl linked dimer **3d**: IR (KBr) 3405, 1702 cm^{-1}; ^1H NMR ($(CH_3)_2CO$), 300 MHz) δ 8.58 (s, 1H), 8.45–7.85 (m 24H), 8.17 (dd, $J=7.7$, 1.7 Hz, 2H), 7.61 (t, $J=7.9$ Hz) δ 7.12 (s, 4H), 7.02 (s, 4H), 6.96 (s, 4H), 6.94 (s, 4H), 6.40 (d, $J=7.9$ Hz, 2H), 6.00 (appar t, $J=9.6$ Hz, 2H), 5.65 (appar t, $J=9.6$ Hz, 2H), 5.62 (appar t, $J=9.6$ Hz, 2H), 4.61–4.49 (m, 4H), 4.39 (dd, $J=12.8$, 4.5 Hz, 2H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 166.0, 165.5, 165.4, 165.2, 163.8, 145.7, 145.6, 144.5, 139.1, 138.9, 138.7, 136.9, 135.3, 135.2, 130.3. 130.1, 121.1, 120.3, 120.9, 120.3, 120.2, 120.1, 110.0, 109.9, 109.8, 93.7, 73.8, 72.8, 71.5, 68.9, 62.4; MS (+FAB) 1706 (M^+, 14); HRFABMS calcd for $C_{76}H_{58}O_{46}$: 1706.2199. Found: 1706.2221.

Biphenyl-4,4′-dicarbonyl dichloride (6e).[22] A solution of biphenyl-4,4′-dicarboxylic acid (200 mg, 0.83 mmol) in 15 mL of CH_2Cl_2 was stirred with oxalyl chloride (0.80 mL, 9.14 mmol) and one drop of DMF under Ar for 18 h. Removal of solvents in vacuo afforded 211 mg (93%) of biphenyl-4,4′-dicarbonyl dichloride (**6e**) as a yellow solid: IR (CH_2Cl_2) 1781 cm^{-1}; ^1H NMR ($CDCl_3$, 360 MHz) δ 8.25 (d, $J=8.2$ Hz, 4H), 7.78 (d, $J=8.7$ Hz, 4H); ^{13}C NMR ($CDCl_3$, 90 MHz) δ 167.9, 145.8, 133.2, 132.1, 127.9.

1,1′-*O*-2,2′,3,3′,4,4′,6,6′-Tetrakis(3,4,5-tribenzyloxybenzoyl)-β,β′-D,D′-glucopyranosylbiphenyl-4,4′-diester (8e). To a solution alcohol **7** (440 mg, 0.24 mmol) in 3 mL of dry THF was added triethylamine (100 μL, 0.72 mmol) followed by diacid chloride **6e** (33 mg, 0.12 mmol). The resulting mixture was heated at reflux for 40 h. The reaction mixture was treated with 1 N HCl and extracted with EtOAc. The organic layer was washed sequentially with H_2O and brine, and then dried over Na_2SO_4. After removal of the solvents in vacuo, the product was purified by flash chromatography using 3:5:12 EtOAc/benzene/hexane to afford 80 mg (17%) of benzylated dimer **8e**: IR ($CDCl_3$) 1732 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz, β,β′ isomer) δ 8.08 (d, $J=8.3$ Hz, 4H), 7.57 (d, $J=8.6$ Hz, 4H), 7.47–7.18 (m, 136H), 6.35 (d, 2H, $J=8.3$ Hz, 2H), 6.07 (appar t, 2H, $J=9.6$ Hz), 5.87 (dd, $J=9.4$, 8.3 Hz, 2H), 5.76 (appar t, $J=9.6$ Hz, 2H), 5.15–4.80 (m, 50H), 4.49–4.43 (m, 2H), 4.36 (dd, $J=11.9$, 6.2 Hz, 2H); ^{13}C NMR ($CDCl_3$ 75 MHz, β, β′ isomer) δ 168.6, 165.6, 165.5, 165.0, 164.2, 152.6, 152.5, 152.4, 144.6, 143.0, 142.5, 137.4, 137.3, 137.2, 136.6, 136.3, 136.2, 133.4, 132.6, 128.8, 128.7, 128.7, 128.6, 128.5, 128.4, 128.3, 128.2, 128.1, 128.0, 127.8, 127.6, 127.5, 123.6, 123.5, 109.3, 109.1, 109.0, 75.1, 75.1, 71.2, 71.1, 71.0, 69.3, 63.1. Anal. calcd for $C_{250}H_{206}O_{46}$: C, 76.10, H, 5.23. Found: C, 75.85, H, 5.31.

1,1′-*O*-2,2′,3,3′,4,4′,6,6′-Tetrakis(3,4,5-trihyroxybenzoyl)-β,β′-D,D′- glucopyranosylbiphenyl-4,4′-diester (3e). By use of general procedure B, dimer **8e** (50 mg, 0.013

mmol) was hydrogenated using 13 mg (0.012 mmol) of Pd/C in 4 mL of THF to furnish 20 mg (87%) of biphenyl linked dimer **3e**: IR (KBr) 3448, 1702 cm^{-1}; ^1H NMR ($(CD_3)_2CO$, 300 MHz) δ 8.28–8.08 (m, 24H), 8.05 (d, $J=8.7$ Hz, 4H), 7.79 (d, $J=8.3$ Hz, 4H), 7.14 (s, 4H), 7.03 (s, 4H), 7.00 (s, 4H), 6.95 (s, 4H), 6.40 (d, $J=8.3$ Hz, 2H), 6.02 (appar t, $J=9.6$ Hz, 2H), 5.71–5.61 (m, 4H), 4.61–4.52 (m, 4H), 4.39 (dd, $J=12.2$, 4.7 Hz, 2H); ^{13}C NMR ($(CD_3)_2CO$, 90 MHz) δ 167.3, 166.8, 166.7, 166.5, 165.7, 146.9, 146.8, 146.5, 140.3, 140.1, 139.9, 138.9, 132.3, 132.3, 130.2, 129.4, 122.4, 121.6, 121.5, 121.4, 111.2, 111.1, 111.1, 94.7, 75.1, 74.0, 72.8, 70.2, 63.7; MS (+FAB) 1782 (M^+); HRFABMS calcd for $C_{82}H_{62}O_{46}$: 1782.2512. Found: 1782.2507; $[\alpha]_D^{20}=-8.3$ (CH_3OH).

1,1′-*O*-2,2′,3,3′,4,4′,6,6′-Tetrakis(3,4,5-tribenzyloxybenzoyl)-β,β′-D,D′-glucopyranosylpimelate (8b). By use of general procedure A, pimeloyl chloride (13 μL, 0.079 mmol) was coupled to alcohol **7** (300 mg, 0.16 mmol) and purified by flash chromatography to afford 140 mg (45%) of benzylated dimer **8b** (mixture of α,α′, β,β′, and α,β′ anomers): IR ($CDCl_3$) 1730 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz, β,β′ isomer) δ 7.43–7.18 (m, 136H), 6.09 (d, $J=7.9$ Hz, 2H), 5.94 (appar t, $J=9.8$ Hz, 2H), 5.69–5.60 (m, 4H), 5.13–4.72 (m, 50H), 4.32–4.27 (m, 4H); ^{13}C NMR ($CDCl_3$, 90MHz) δ 171.5, 165.6, 165.5, 165.0, 165.0, 164.7, 152.6, 152.5, 152.4, 143.3, 143.1, 143.1, 143.0, 142.7, 142.6, 137.5, 137.3, 136.7, 136.4, 136.4, 136.2, 128.5, 125.5, 128.4, 128.3, 128.3, 128.2, 128.1, 128.1. 128.0, 127.8, 127.6, 124.5, 123.7, 123.6, 109.2, 109.1, 921, 75.1, 75.1, 71.2, 71.1, 71.0, 69.9, 63.1, 33.6, 28.1, 23.9; MS (+FAB) 3861 (MH^+, 50).

1,1′-*O*-2,2′,3,3′,4,4′,6,6′-Tetrakis(3,4,5-trihyroxybenzoyl)-β,β′-D,D′-glucopyranosylpimelate (3b). By use of general procedure B, dimer **8b** (140 mg, 0.036 mmol) was hydrogenated using 32 mg (0.03 mmol) of Pd/C in 5 mL of THF to afford 60 mg (97%) of pentyl linked dimer **3b**: IR (KBr) 3422, 1718 cm^{-1}; ^1H NMR ($(CD_3)_2CO$, 300 MHz) δ 8.17–7.45 (m, 24H), 7.06 (s, 4H), 6.91 (s, 4H), 6.90 (s, 4H), 6.82 (s, 4H), 6.05 (d, $J=8.3$ Hz, 2H), 5.79 (appar t, $J=9.6$ Hz, 2H), 5.48 (appar t, $J=9.8$ Hz, 2H), 5.32 (dd, $J=9.8$, 8.3 Hz, 2H), 4.28–4.23 (m, 2H); ^{13}C NMR ($(CD_3)_2CO$, 75 MHz) δ 171.3, 165.8, 165.2, 165.0, 145.5, 145.4, 145.4, 145.3, 138.9, 138.8, 138.6, 138.5, 120.8, 120.0, 119.9, 119.8, 109.7, 109.6, 109.5, 109.5, 92.2, 73.3, 72.6, 71.2, 68.6, 62.2, 33.6, 28.2, 24.4; MS (+FAB) 1701 (MH^+); HRFABMS calcd for $C_{75}H_{64}O_{46}$: 1700.2669. Found: 1700.2577; $[\alpha]_D^{20}=+53.3$ (CH_3OH).

2,3′-Oxy-di-benzoic acid (5).[25] Carboxylic ester **4**[23] (0.118 g, 0.412 mmol) was combined with $LiOH\cdot H_2O$ (0.105 g, 2.50 mmol) in 3 mL of MeOH and 1 mL of H_2O. The reaction mixture was heated to reflux under Ar for 5 h. The solution was cooled to rt, acidified with 1 N HCl, and extracted with EtOAc. The organic layer was washed with brine and dried over $MgSO_4$. After filtration and removal of solvents in vacuo, 0.099 g (93%) of diaryl ether diacid **5** was collected as a white powder: ^1H NMR ($(CD_3)_2CO$, 300 MHz) δ 9.5 (br s, 2H), 8.00 (dd, $J=7.9$ Hz, 1.9 Hz, 1H), 7.75 (appar t of d, $J=7.8$, 1.2, 1H), 7.64 (ddd, $J=8.2$, 7.5, 1.8 Hz, 1H), 7.53–7.46 (m, 2H), 7.35 (appar d of t, $J=7.6$, 1.1 Hz, 1H), 7.21 (ddd, $J=8.2$, 2.6, 1.0 Hz, 1H), 7.14 (dd, $J=8.2$, 1.0 Hz, 1H); ^{13}C NMR ($(CD_3)_2CO$, 75 MHz) δ 166.6, 166.0, 158.8, 155.6, 134.4, 132.6, 132.5, 130.3, 125.0, 124.7, 124.2, 122.5, 122.3, 118.4.

2,3′-Oxy-di-benzoyl chloride (6a).[25] Oxalyl chloride (0.058 μL, 0.66 mmol) was added slowly dropwise to a suspension of diacid **5** (0.057 g, 0.22 mmol) in 2 mL dry CH_2Cl_2 and 1 drop DMF. The suspension gradually turned to a clear yellow solution. The reaction mixture stirred under Ar for 3 h. The solvent was removed in vacuo to yield 0.063 g (97%) of diaryl ether diacid chloride **6a** as a yellow oil: IR ($CDCl_3$) 1758 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 8.19 (dd, $J=8.3$, 1.7 Hz, 1 Hz) 7.93–7.90 (m, 1H), 7.68 (appar t, $J=2.1$ Hz, 1H), 7.66–7.60 (m, 1H), 7.52 (appar t, $J=8.1$ Hz, 1H), 7.36–7.31 (m, 2H), 7.00 (dd, $J=8.3$, 0.7 Hz, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 167.6, 163.9, 156.9, 155.4, 136.1, 135.0, 134.3, 130.5, 126.8, 125.8, 125.4, 124.5, 120.5, 120.4.

1,1′-*O*-2,2′,3,3′,4,4′,6,6′-Tetrakis(3,4,5-tribenzyloxybenzoyl)-β,β′-D,D′-glucopyranosyl-(2,3′-oxy-di-benzoate) (8a). By use of general procedure A, diacid chloride **6a** (31 mg, 0.11 mmol) was coupled to alcohol **7** (373 mg, 0.20 mmol) with triethylamine (88 μL, 0.63 mmol) in 2.5 mL of CH_2Cl_2, and the crude product was purified by flash chromatography to afford 147 mg (37%) of benzylated dimer **8a**: IR ($CDCl_3$) 1731 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.97 (dd, $J=7.9$, 1.5 Hz, 1H), 7.76–7.73 (m, 1H), 7.66–7.65 (m, 1H), 7.41–7.15 (m, 138H), 7.13–7.00 (m, 2H), 6.72 (d, $J=8.3$ Hz, 1H), 6.29 (d, $J=7.9$ Hz, 1H), 6.26 (d, $J=7.9$ Hz, 1H), 6.04 (appar t, $J=9.8$ Hz, 1H), 5.97 (appar t, $J=9.8$ Hz, 1H), 5.82–5.68 (m, 4H), 5.10–4.9 (m, 48H) 4.93–4.72 (m, 2H), 4.37–4.27 (m, 4H); ^{13}C NMR ($CDCl_3$, 90 MHz) δ 165.5, 165.4, 165.0, 164.9, 164.8, 164.8, 164.0, 162.8, 162.8, 162.4, 157.3, 156.7, 152.5, 152.5, 152.4, 152.4, 143.1, 143.1, 143.0, 143.0, 143.0, 142.5, 142.5, 137.5, 137.5, 137.4, 137.3, 137.3, 136.7, 136.6, 136.4, 136.4, 136.3, 136.3, 134.9, 132.6, 130.2, 130.2, 128.5, 128.4, 128.3, 128.3, 128.2, 128.1, 128.0, 127.9, 127.8, 127.5, 124.8, 124.5, 124.5, 123.7, 123.7, 123.6, 120.6, 120.3, 119.5, 109.3, 109.1, 109.1, 92.9, 92.5, 77.2, 75.1, 75.1, 73.6, 73.4, 73.1, 71.2, 71.1, 71.1, 71.0, 69.8, 69.7, 67.5, 63.2, 62.9.

1,1′-*O*-2,2′,3,3′,4,4′,6,6′-Tetrakis(3,4,5-trihyroxybenzoyl)-β,β′-D,D′-glucopyranosyl-(2,3′-oxy-di-benzoate) (3a). By use of general procedure B, dimer **8a** (131 mg, 0.033 mmol) was hydrogenated using 17 mg (0.016 mmol) of Pd/C in 2.5 mL of THF to afford 70 mg (99%) of diaryl ether linked dimer **3a**: ^1H NMR ($CDCl_3$, 360 MHz) δ 8.18 (m, 24H), 7.91 (m, 1H), 7.71 (d, $J=7.8$ Hz, 1H), 7.60–7.53 (m, 2H), 7.40–7.34 (m, 2H), 7.28–7.24 (m, 2H), 7.19 (s, 4H), 7.06 (s, 2H), 7.04 (s, 2H), 7.02 (s, 2H), 6.98 (s, 2H), 6.97 (s, 2H), 6.94 (s, 2H), 6.39 (d, $J=8.2$ Hz, 1H), 6.35 (d, $J=8.2$ Hz, 1H), 6.04 (appar t, $J=9.8$ Hz, 1H), 5.97 (appar t, $J=9.6$ Hz, 1H), 5.71–5.56 (m, 4H), 4.61–4.40 (m, 6H); ^{13}C NMR ($(CD_3)_2CO$, 90 MHz) δ 166.0, 165.4, 165.2, 165.1, 165.1, 164.1, 164.1, 162.6, 157.9, 156.6, 145.6, 145.5, 145.4, 138.9, 138.7, 138.6,

135.5, 132.3, 130.8, 130.7, 124.9, 124.6, 124.2, 121.8, 121.3, 121.3, 120.9, 119.6, 109.8, 109.8, 109.7, 93.4, 93.0, 73.6, 73.5, 72.8, 72.6, 71.3, 71.2, 68.8, 68.7, 62.3, 62.2; MS (+MALDI) 1822 (MNa^+); $[\alpha]_D^{20} = +$ 43.8 (CH_3OH).

Immunological assays, general. LPS (*Escherichia coli* 055:b5 phenol extract), Ficoll-Histopaque-1077, Fetal Bovine Serum (FCS), gentamicin (10 mg/mL), L-glutamine (200 mM), Dextran B-512 (ave M_r 580,000), and Trypan Blue stain (0.4%) were purchased from Sigma. Hanks' Balanced Salt Solution 1X, with phenol red (HBSS) and RPMI 1640 1X (Mediatech) were purchased from Fisher Scientific. A complete culture medium (cRPMI) was prepared by addition of 10 mL of gentamicin solution, 10 mL of L-glutamine solution, and 100 mL of FCS to 1 L of RPMI 1640. Human and rat TNF-α and IL-1β ELISA kits were purchased from R&D Systems, Minneapolis, MN, USA and Biosource International, Camarillo, CA, USA.

Isolation of h-PBMCs. Fresh heparinized blood was obtained from healthy human subjects (ages 20–34 years). PBMCs were isolated from reported procedures[26] (see also ref 27). The cells were counted and the viability was determined by Trypan Blue exclusion (typically, viability exceeded 95%). Experiments were conducted in a 5% CO_2, 37 °C humidified incubator for the indicated time period.

h-PBMCs, Dimer dose response data. 0.5 mL Samples of PBMC suspension (2×10^6 cells/mL in cRPMI) were incubated for 24 h with the appropriate amounts of a dimer (**3a**–**e**) or LPS stock solution in HBSS to furnish the desired concentration range. Each concentration value was run in triplicate, and blank runs ensured that (bacterial) contamination did not complicate the experiments. After 24 h, culture supernatants were harvested and analyzed for TNF-α by Enzyme Linked Immunosorbent Assay (ELISA). The cytokine values reported are mean ± SEM.

h-PBMCs, Dimer inhibition data. 0.5 mL Samples of PBMC suspension (2×10^6 cells/mL in RPMI 1640) were incubated with 5 or 10 μg/mL of LPS in HBSS for 45 min. At this time, the non-control wells were treated with the appropriate amount of a dimer (**3b**–**e**) stock solution in HBSS to furnish the desired concentration range. Each concentration value was run in triplicate, and blank runs ensured that (bacterial) contamination did not complicate the experiments. After 8 h, culture supernatants were harvested and analyzed for TNF-α by ELISA. The cytokine values reported are mean ± SEM.

Rat inhibition data. Chronically catheterized, conscious, unrestrained rats 1–4 were given a primed constant infusion of dimer **3b** or **3e** (20 mg/rat, 5 mg as initial injection + 5 mg/h as constant infusion). DMSO was used to aid in solubility of the dimers. 10 min after the initial injection, the animals were treated with a 1 mg/kg dose of LPS (*E. coli*, 026:B6, Difco, Detroit, MI, USA). Rats 5–8 were administered LPS + DMSO only, and control rat 9 was given saline only. Blood plasma samples (and hemodynamic measurements) were collected prior to the start of the study and at 90 and 180 min and analyzed for TNF-α and IL-1β by ELISA. The cytokine values reported are mean ± SEM.

Acknowledgements

We thank the National Institutes of Health [GM 35727 (KSF) and GM 38032 (CHL)] for financial support of this work.

References and Notes

1. Edwards, C. K., III; Borcherding, S. M.; Zhang, J.; Borcherding, D. B. In *Xenobiotics and Inflammation*; Schook, L. B. and Laskin, D. L., Eds.; Academic: New York, 1994, p 97.
2. Beutler, B. *J. Invest. Med.* **1995**, *43*, 227.
3. Old, L. J. *Science* **1985**, *230*, 630.
4. Lynn, W.; Golenbock, D. *Immunol. Today* **1992**, *13*, 271.
5. Christ, W. J.; Asano, O.; Robidoux, A. L. C.; Perez, M.; Wang, Y.; Dubic, G. R.; Gavin, W. E.; Hawkins, L. D.; McGuinness, P. D.; Mullarkey, M. A.; Lewis, M. D.; Kishi, Y.; Kawata, T.; Bristol, J. R.; Rose, J. R.; Rossignol, D. P.; Kobayashi, S.; Hishinuma, I.; Kimura, A.; Asakawa, N.; Katayama, K.; Yamatsu, I. *Science* **1995**, *268*, 80.
6. Newton, R. C.; Decicco, C. P. *J. Med. Chem.* **1999**, *42*, 2295.
7. Black, R. A.; Bird, T. A.; Mohler, K. M. *Annu. Rep. Med. Chem.* **1997**, *32*, 241.
8. Niwayama, S.; Turk, B. E.; Liu, J. O. *J. Med. Chem.* **1996**, *39*, 3044.
9. Sampaio, E. P.; Sarno, E. N.; Galilly, R.; Cohn, Z. A.; Kaplan, G. *J. Exp. Med.* **1991**, *173*, 699.
10. Borcherding, D. R.; Peet, N. P.; Munson, H. R.; Zhang, H.; Hoffman, P. F.; Bowlin, T. L.; Edwards, I. *C. K. J. Med. Chem.* **1996**, *39*, 2615.
11. Feldman, K. S., Sahasrabudhe, K., Lawlor, M. D., Wilson, S. L., Lang, C. H.; Scheuchenzuber, W. J. *Bioorg. Med. Chem. Lett.*, **2001**, *11*, 1813.
12. Haslam, E. *Practical Polyphenolics*; Cambridge University Press: Cambridge, 1998.
13. Feldman, K. S.; Sahasrabudhe, K.; Quideau, S.; Hunter, K. L.; Lawlor, M. D. In *Plant Polyphenols 2: Chemistry, Biology, Pharmacology, Ecology*; Kluwer Academic/Plenum: New York, 1999.
14. Okuda, T.; Yoshida, T.; Hatano, T. In *Progress in the Chemistry of Organic Natural Products*; Herz, W., Kirby, G. W., Moore, R. E., Steglich, W., Tamm, C., Eds.; Springer: New York, 1995; Vol. 66, p 1, and references cited therein.
15. Kuby, J. *Immunology*, 3rd ed.; W. H. Freeman: New York, 1997; Chapter 13.
16. Feldman, K. S.; Sahasrabudhe, K.; Smith, R. S.; Scheuchenzuber, W. J. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 985.
17. Ulevitch, R. J.; Tobias, P. S. *Ann. Rev. Immunol.* **1995**, *13*, 437.
18. Beutler, B. *Curr. Opin. Immunol.* **2000**, *12*, 20.
19. Baxter, N. J.; Lilley, T. H.; Haslam, E.; Williamson, M. P. *Biochemistry* **1997**, *36*, 5566.

20. Feldman, K. S.; Sahasrabudhe, K. *J. Org. Chem.* **1999**, *64*, 209.
21. Bols, M.; Hansen, H. C. *Acta Chem. Scand.* **1993**, *47*, 818.
22. Burdett, K. A. *Synthesis* **1991**, 442.
23. Nagai, Y.; Irie, A.; Nakamura, H.; Hino, K.; Uno, H.; Nishimura, H. *J. Med. Chem.* **1982**, *25*, 1065.
24. An initial kinetics experiment determined that optimal inhibition was achieved after 8 h.
25. Anschütz, R.; Stoltenhoff, W.; Voeller, F. *Chem. Ber.* **1925**, *58*, 1736.
26. Murayama, T.; Kishi, N.; Koshiura, R.; Takagi, K.; Furukawa, T.; Miyamoto, K. *Anticancer Res.* **1992**, *12*, 1471.
27. Kanof, M. J.; Smith, P. D. I. In *Current Protocols in Immunology*; Coligan, J. E., Kruisbeck, A. M., Margulies, D. M., Eds.; Wiley Interscience: New York, 1991; Vol. 1, Unit 7.1.