Published in final edited form as:

Chembiochem. 2014 January 24; 15(2): 250–258. doi:10.1002/cbic.201300588.

Modulation of CD14 and TLR4.MD-2 activities by a synthetic lipid A mimetic

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Abstract

Monosaccharide lipid A mimetics composed by a glucosamine core linked to two fatty acid chains and bearing one or two phosphates have been synthesized. While compounds **1** and **2**, with one phosphate group, were practically inactive in inhibiting LPS-induced TLR4 signaling and cytokine production in HEK-blueTM cells and murine macrophages, compound **3** with two phosphates was found to be active in efficiently inhibiting TLR4 signal in both cell types. The direct interaction of molecule **3** with MD-2 co-receptor has been investigated by means of NMR and molecular modeling/docking analysis. This compound also interacts directly with CD14 receptor, stimulating its internalization by endocytosis. Experiments on macrophages show that the effect on CD14 reinforces the activity on MD-2.TLR4, because compound **3** activity is higher when CD14 is important for TLR4 signaling i.e, at low LPS concentration. The dual MD-2 and CD14 targeting, accompanied by good solubility in water and lack of toxicity, suggests the use of monosaccharide **3** as a lead compound to develop drugs directed against TLR4-related syndromes.

Keywords

drugs; carbohydrates; bioorganic chemistry; NMR; molecular modeling

Introduction

Activation of Toll-like Receptor 4 (TLR4) and subsequent intracellular signaling in response to minute amounts of circulating endotoxins (Gram-negative bacterial lipopolysaccharides, LPS), results in the rapid triggering of pro-inflammatory processes necessary for optimal host immune responses to invading Gram-negative bacteria in mammals.^[1] TLR4 does not bind directly to LPS, and TLR4 activation by endotoxin is a complex event, involving the participation of other LPS-binding proteins, namely LBP, CD14 and MD-2 and ending with the formation of the activated (TLR4.MD-2.LPS)₂ complex.^[2] In particular, CD14 was the first identified Pattern Recognition Receptor (PRR) that binds directly to LPS,^[3] and chaperones the formation of the (TLR4.MD-2.LPS)₂ complex.^[4] At low endotoxin concentration CD14 has a fundamental role in assisting the formation of the signaling complex and the consequent initiation of the MyD88-dependent pathway leading to NF-κB activation. Vice-versa CD14 is not indispensable for the activation of this pathway when LPS is more concentrated.^[5] CD14 is also required for endotoxin-induced TLR4 endocytosis^[6] and relocalization of the entire LPS receptor complex in the endosome where a second signaling pathway, namely the TRIF-dependent pathway, leading to a second wave of NF-κB and IRF3 activation and inflammatory cytokine production initiates. It has been recently observed that TLR4 antagonists, such as Eritoran,^[7] lipid IVa, and cationic glycolipids,^[8] strongly interact with CD14 and inhibit the formation of CD14-endotoxin complex. Excessively potent and deregulated TLR4 activation and signaling causes serious systemic syndromes such as fatal septic shock, associated with a high mortality (20–30%),^[9] and organ-specific syndromes. CD14-dependent TLR4 activation in central nervous system (CNS) by endogenous factors has been recently related to a wide array of inflammatory neurological diseases such as amyotrophic lateral sclerosis (ALS),^[10] neuropathic pain^[11] and Alzheimer disease (AD).^[12] Efficient and selective TLR4 antagonists with a chemical structure simpler than lipid A are therefore required to develop new drugs with a wide array of medical and pharmacological settings (from sepsis to CNS pathologies).^[13] The lipid A moiety of LPS, which anchors LPS to the outer membrane of Gram-negative bacteria, is responsible for the immunostimulatory activity of LPS.^[14, 15] Lipid A consists of a 1,4-β-diphosphorylated di-glucosamine backbone to which variable lengths and numbers of fatty acid (FA) acyl chains are covalently linked.^[15] The number and structure of acyl chains, as well as the two phosphate groups, determine the agonistic activity of lipid A. Lipid X (Figure 1), a biosynthetic precursor of lipid A, whose structure corresponds to the reducing GlcNAc monosaccharide of *E. coli* lipid A, blocks LPS-induced septic shock and TLR4-dependent human neutrophils priming.^[16] Due to its anti-endotoxic activity^[17] lipid X has been considered a simplified monosaccharide scaffold for the development of TLR4 agonists and antagonists.

In this paper, we present the synthesis and the biological characterization of monosaccharides **1–3**: compound **1** corresponds to a lipid X mimetic with an α-anomeric phosphate, while **2** has a phosphate ester in C-4 position and **3** is phosphorylated on both C-1 and C-4 positions. Natural lipid A and lipid X have the (C-1) anomeric phosphate exclusively in the α configuration and the stereochemistry at the anomeric bond is very important for biological activity.^[15] Accordingly, we introduced anomeric (C-1) phosphate

esters in compounds **1** and **3** using stereoselective reactions leading exclusively to the α configuration. Extensive structure-activity studies are available on lipid X mimetics formed by a GlcNac monosaccharide with a C-4 phosphate and acylated in C-2 and C-3 positions with different linear and branched FA chains.^[18, 19] While compounds with two C₁₄ FA acyl chains in C-3 and a linear C₁₄ chain in C-2 have TLR4 agonist activity in human and mouse macrophages, compounds with different acylation patterns (included compound **1**, named GLA-26, with two linear acyl chains) acted as agonists in murine macrophages and antagonist in human monocytes.^[18,20] Compound **2** with a phosphate in C-4 has been described (compound 880.244)^[21] as a very weak TLR4 modulator.^[21, 22] Among all lipid X synthetic analogues, the diphosphoryl lipid X (Scheme 1) showed the most potent antagonist activity on both murine macrophages and human monocytes.^[23] Compound **3** closely resembles to diphosphoryl lipid X but its structure is further simplified by the removal of C-3 hydroxyls on FA chains.

Results and Discussion

Synthesis of lipid X mimetics 1–3

Compounds **1–3** were synthesized using a divergent strategy, starting from the common intermediate **4**,^[24] obtained from commercial D-glucosamine hydrochloride (Scheme 2). Compound **4** has been treated with PPh₃ in THF/H₂O to transform the azido group into amine (compound **5**), then acylated in C-2 and C-3 positions using myristic acid in the presence of the condensing agent 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), thus affording **6**. Monosaccharide **6** was deprotected on anomeric (C-1) position using tetrabutylammonium fluoride (TBAF) and AcOH to obtain **7**, which was phosphorylated using tetrabenzoyldiphosphate in the presence of lithium bis(trimethylsilyl)amide affording the α -anomer **8** exclusively. Catalytic hydrogenation with Pd/C allowed simultaneous removal of *p*-methoxybenzylidene and benzyl groups on phosphate, affording compound **1**. Alternatively, the benzylidene protecting group of compound **6** was reductively opened by treatment with NaCNBH₃ in dry THF to obtain compound **9** protected as *p*-methoxybenzyl (PMB) ether on C-4 position. Compound **9** was phosphitylated using dibenzyl *N,N*-diisopropylphosphoramidite and imidazolium triflate, then oxidized to phosphate with *m*-chloroperbenzoic acid to obtain **10**. Compound **10** was deprotected on C-1 using TBAF and AcOH affording **11**, then catalytic hydrogenation with Pd/C allowed to obtain compound **2**. To have access to compound **3**, compound **6** was treated with NaCNBH₃ with regioselective opening of benzylidene ring and formation of C-6 PMB ether. This reaction was quenched by adding an acidic solution (HCl 1M in dioxane at pH1.5 for 1h), that promoted the removal of the anomeric TBDMS protective group, thus obtaining compound **12** in one reaction step. The double phosphorylation on C-1 (stereoselective) and C-4 positions using phosphoramidite plus oxidation method, followed by catalytic hydrogenation afforded monosaccharide **3**. Compounds **1**, **2** and **3** were recovered as triethylammonium salts after hydrogenation, then treated with Amberlite IR 120 Na⁺ exchange resin to change the counterion. The final compounds used for biological characterization contained sodium counterion for phosphate groups.

Inhibition of LPS-Induced, TLR4-dependent NF- κ B activation in HEK-blue™ cells

The ability of molecules **1**, **2** and **3** to interfere with LPS-triggered TLR4 activation was first investigated in HEK-blue™ cells. HEK-Blue™ cells are HEK293 cells stably transfected with human TLR4, MD-2, and CD14 genes. In addition, HEK-blue™ cells stably express a secreted Alkaline Phosphatase (sAP) produced upon activation of NF- κ B. LPS binding activates TLR4 and NF- κ B leading to sAP secretion, which is detected by an alkaline phosphatase substrate in cell culture media. When supplied alone, compounds **1–3** were unable to stimulate TLR4-dependent sAP production in a range of concentrations between 0 and 50 μ M, thus confirming the lack of any agonist activity for the three monosaccharides on human TLR4. Cells were then pre-treated with increasing concentrations of synthetic monosaccharides (from 0 to 50 μ M) and then stimulated with *E. coli* O55:B5 LPS (100 ng/mL). In this concentration range compounds **1** and **2** were weakly active in inhibiting LPS-stimulated TLR4 signaling, while **3** was active. The experiment was also run for molecule **3** at two LPS concentrations, 10 ng/mL and 1 μ g/mL, by administering LPS 30 min after the pretreatment with compound **3**. At both these LPS concentrations the % activation of HEK cells reached similar values, thus indicating that the lower concentration (10 ng/mL) also saturated the signal corresponding to TLR4-dependent NF- κ B stimulation (the 100% of vertical scale, Figure 2). Compound **3** turned out to be more active as antagonist at low LPS concentration (10 ng/mL), with a calculated IC₅₀ of 0.46 μ M, while when LPS was more concentrated, the IC₅₀ raised at 3.42 μ M (Figure 1). As a negative control a HEK-293 cell line (InvivoGen) transfected with the same plasmids as HEK-blue™ but without TLR4, MD-2 and CD14 genes was used and no effect was observed (data not shown). The toxicity of all compounds was assessed by MTT test and all compounds showed no or very limited toxicity up to the highest concentration tested (50 μ M, Supp Info).

Inhibition of LPS-Induced TLR4 activation in murine macrophages

The ability of molecules **1–3** to modulate the LPS-stimulated TLR4 pathway was further investigated in bone marrow-derived murine macrophages (BMM Φ). TNF- α production was taken as read out of TLR4-MyD88 pathway activation. BMM Φ from wild type and CD14^{-/-} mice were treated with increasing concentrations (0–50 μ M) of compounds **1–3** in DMEM +BSA 0.03% in the presence or absence of LPS. Two LPS concentrations were tested: 10 ng/mL and 1 μ g/mL, and LPS was administered 30 min after the pretreatment with synthetic compounds. The LPS-induced TNF- α production after one night incubation was assessed by ELISA test (Figure 2). As expected, high concentration of smooth LPS activated TLR4 signaling also in the absence of CD14,^[5] while at low LPS concentration the signal was absent in CD14-defective cells. Compound **1** was inactive in both cell types, molecule **2** showed a weak antagonist effect on wt macrophages at low LPS concentration. Compound **3** showed a dose-dependent LPS antagonistic activity, in wt cells at low LPS concentration, and in CD14^{-/-} at high LPS concentration (Figure 2). The antagonist activity on both wt and CD14-defective cells, suggests that molecule **3** competes with LPS for the interaction with both CD14 and MD-2.TLR4 complex.

Compound 3 selectively stimulates endocytosis of CD14 (and not of MD-2.TLR4 complex)

Since CD14 favors the activation of the TLR4-MyD88 pathway at low LPS doses, and compounds **2** and **3** are more active as inhibitors at low LPS concentrations, we evaluated whether CD14 could be directly targeted by synthetic compounds. We analyzed the capacity of molecules **1–3** to induce CD14 and MD-2.TLR4 complex internalization on BMM Φ . As a matter of fact, after LPS or lipid A binding, CD14 is efficiently internalized together with the entire LPS receptor complex and this process has been demonstrated to be directed by CD14 in a TLR4-independent manner.^[6] BMM Φ were incubated with compounds **1–3** at a concentration of 10 μ M, and the amount of CD14 remaining at the cell surface analyzed by flow cytometry over-time. Molecule **3**, showing the best antagonistic activity, was also capable of efficiently inducing CD14 internalization, while molecules **1** and **2** did not show any effect on CD14 surface expression (Figure 3). Interestingly MD-2.TLR4 complex was not internalized after molecules **1–3** exposure (Figure 3). These results suggest that molecule **3**, an antagonist of the TLR4 signal pathway, can directly interact with CD14 causing its internalization.

NMR binding experiments: interaction between synthetic molecule 3 and MD-2

The binding of monosaccharide **3** to MD-2 receptor was then investigated by means of NMR techniques in solution. Because the tendency of compound **3** to form stable aggregates at the concentration required for NMR measurements (150–300 μ M), it turned out to be impossible to detect ligand-protein interaction by Saturation Transfer Difference (STD) experiments. However, it was possible to record good quality ¹H-NMR spectra of **3**, of MD-2 and of a **3**:MD-2 mixture (ratio 5:1, at a 150 μ M ligand concentration, Figure 5). Fittingly, selective attenuation of the signals corresponding to the fatty acid C₁₄ chain protons of **3** was observed upon addition of MD-2 to the monosaccharide sample solution (Figure 4). Although broadening of all the resonance signals was detected, the decrease in signal intensities was significantly higher for those hydrogens belonging to the FA aliphatic chains, in particular for the signals of the Ω methyl groups and for those of the contiguous CH₂ moieties, followed by the rest of the chain hydrogens. In contrast, the intensity of the signals corresponding to the hydrogens on the sugar ring resulted practically unaltered. The experimentally observed reduction in intensity, due to specific line broadening of these signals, arises from the change in transverse relaxation time of these signals. This dramatic change likely arises from the existence of interaction between **3** and MD-2, precisely focused at the aliphatic side chain region. In fact, the FA chains-MD-2 interactions outlined here are reminiscent of those previously observed for positively charged amphiphiles.^[8] These data suggest the occurrence of a major interaction of both FA chains of the sugar ligand with the hydrophobic binding cavity of MD-2, as also confirmed by the docking calculations (see below). The exchange process between the free and bound states provides the basis for the increased relaxation rate and the corresponding observed increase of linewidth. Additional features of the interaction were also investigated by using DOSY (Diffusion Ordered SpectroscopY) NMR. First, the aggregation properties of **3** were evaluated by means of DOSY. The DOSY of the free ligand showed a strikingly small diffusion coefficient, corresponding to a high molecular weight species in solution. This evidence can be associated to the fact that molecule **3** forms relatively large aggregates

(Figure S2, Supp. Info.) in water solution. Then, the DOSY spectrum of the **3**:MD-2 mixture (at 5:1 molar ratio) was also recorded. As stated above, the signals of the ligand aliphatic chains were no more visible in the NMR spectrum, strongly suggesting the interaction of this part of the molecule with MD-2. In addition, the observation of a higher diffusion coefficient for the ligand molecule indicated that the existing aggregate for isolated **3** is indeed disrupted (Supp. Info.).

Molecular modeling studies and docking of monosaccharide **3** with CD14 and MD-2

Docking studies were performed using the AutoDock^[25] and AutoDock Vina^[26] programs, applying the protocol described in the experimental section. First, the use of this computational program was validated by docking the natural antagonist lipid IVa, employing the X-ray crystallographic structure of the human MD-2 protein in complex with lipid IVa as starting geometry (PDB 2E59).^[27] Both AutoDock and Vina programs satisfactorily reproduced the crystallographic binding mode, showing the four FA acyl chains inside the lipophilic pocket, and the phosphorylated glucosamine moieties located at the entrance to the cavity (data not shown). Once the docking procedures were validated, the same docking protocol was applied to compound **3**, predicting reasonable binding poses in both proteins, CD14 and MD-2, pointing at this compound as a suitable binder. Docked theoretical MD-2 binding energies for compounds **1** and **2** were significantly higher than that of compound **3** in at least 8 kJ/mol (data not shown), thus pointing to a more favorable binding process for **3**. Analysis of the AutoDock and AutoDock Vina docked binding poses in MD-2 showed the ability of compound **3** to bind in two different fashions, with close predicted binding energies. Most of the best docked solutions corresponded to binding poses with the two FA chains deeply confined inside the MD2 pocket, similarly to lipid IVa. One of the FA chains establishes hydrophobic contacts and CH- π interactions with Leu74, Phe76, Phe104, and Ile117, in a similar way to the equivalent FA chain present in lipid IVa (Figure 5A). The second FA chain is directed into the region delimited by Ile52, Leu54, Phe121, Ile124, Tyr131, and Ile153, a sub-pocket also occupied by a FA chain in the complex with lipid IVa. In few cases, results from docking showed a second binding mode with one FA chain going out towards Val82, and placed over Ile124 (Figure 5B). Interestingly, this Ile124 moves “up” in the agonist conformation, and its position is occupied by Phe126, thus acting as an agonist/antagonist switch.

Polar interactions were also identified in some of the docked binding poses. One phosphate group participates in hydrogen bonds, for instance with Ser118, and is always placed in the vicinity of Lys58 and/or Lys122, similarly to one of the lipid IVa phosphates. The second phosphate is found in the proximity of positively charged side chains, such as that from Arg96 or, in other docked solutions, exposed to the outside. In addition, either amide CO group or ester CO group from compound **3** was found to establish a hydrogen bond with the Ser120 CO group in some of the docking results. These predicted binding poses are in agreement with the NMR experiments and provide a 3D model for the interaction of the FA chains with MD-2 protein, as well as putative polar interactions involving the phosphate groups. Docking calculations of compound **3** into CD14 were also carried out. In this case, CD14 protein also owns a highly lipophilic wide pocket as MD-2 protein, but with fewer charged residues in the opening portion. Compound **3** showed binding poses presenting both

FA chains inside the pocket, and the polar phosphate groups and sugar placed at the entrance of the cavity (Figure S3, Supp. Info.) thus supporting the experimental evidences of CD14 binding properties for this compound.

Conclusion

With the aim to obtain TLR4 antagonists with a chemical structure simpler than lipid A, mono- and diphosphate lipid X analogues were synthesized (compounds **1–3**). Monosaccharides **1** and **2** with one phosphate group linked respectively to anomeric (C-1) and C-4 positions, showed weak antagonism in HEK-blueTM cells and macrophages. Monosaccharide **3**, a diphosphorylated lipid X analogue lacking the C-3 hydroxyls on FA acyl chains, showed to be active in inhibiting in a dose-dependent way the LPS-stimulated NF- κ B activation in HEK-blueTM cells (Figure 1) and LPS-induced TNF- α production in macrophages (Figure 2). Monosaccharide **3** was much more active in inhibiting cytokine production at low (10 ng/mL) than at higher (1 μ g/mL) LPS concentrations (Figure 2). When LPS concentration is low, the CD14-catalyzed extraction of LPS monomers from aggregates in solution and LPS presentation to MD-2.TLR4 complex is essential to TLR4 activation and signaling.^[5] On the other hand, it has been reported that highly concentrated LPS can bind directly to the MD-2.TLR4 complex and activate the TLR4 cascade without need of the CD14 chaperone. The higher activity of compound **3** in the experimental conditions in which CD14 contributes to TLR4 activation, is a first indication that compound **3** probably interferes in both LPS/MD-2.TLR4 and LPS/CD14 interactions. This behavior is reminiscent of a first generation of positively charged monosaccharides we developed that blocked in vitro and in vivo TLR4 activation^[28] by mainly displacing endotoxin from CD14 interaction.^[8] The capacity of molecule **3** to stimulate CD14 internalization (Figure 3) provides further evidence of a direct interaction between the synthetic molecule and CD14. The interaction with MD-2 has been characterized by NMR studies, allowing the identification of the FA acyl chain moieties as the part of compound **3** that directly interacts with MD-2 (Figure 4). Once again in analogy with what observed with synthetic cationic amphiphiles,^[8] the FA acyl chains of the molecule show to interact with MD-2 more strongly than the sugar part. Computational models for this complex have shown that the most stable docked complexes always correspond to binding poses where the FA chains of compound **3** are placed deeply into the MD-2 pocket, in agreement with the NMR observation of FA chains-protein interactions. Additionally, polar interactions of the phosphate groups with the outer polar residues can be identified in some of the binding poses. Overall, our calculations thus provide a theoretical 3D view of the interaction of this compound with MD-2. A docked model for CD14 in complex with compound **3** has also been proposed in accordance to the experimental results.

Monosaccharide **3** is a promising lead for the development of drugs targeting TLR4 activation in a variety of medical settings.^[9–13] This non toxic TLR4 antagonist shows a high water solubility (completely soluble up to 1 mM concentration) in contrast with lipid A and lipid A mimetics previously developed by us and other groups that suffer from poor solubility in aqueous media.^[13] This is an important prerequisite to good bioavailability and favorable pharmacokinetic (distribution) properties. The critical micelle concentrations

(CMC) for compounds **1–3** were determined via pyrene fluorescence measurements.^[29] Compounds **1**, **2** and **3** CMC values are respectively 13, 28 and 9 μM . The TLR4 antagonist activity of compound **3** is observed in cells at concentration values below the CMC (IC_{50} from 0.46 to 3.2 μM). In this concentration range compound **3** is mainly in the monomeric form in solution. On the contrary, at the higher concentrations required for NMR experiments (from 150 to 330 μM) compound **3** is prevalently in form of aggregates in solution. According to the data presented in this study, compound **3** interacts efficiently with both MD-2(.TLR4) and CD14 receptors. Multiple targeting could explain and contribute to molecule's efficacy in blocking TLR4 signal.

Experimental Section

Chemistry

General procedures—All reagents were commercially available and used without further purification unless indicated otherwise. All solvents were anhydrous grade unless indicated otherwise. When dry conditions were required, the reactions were carried out in oven-dried glassware under a slight pressure of argon. Reactions were magnetically stirred and monitored by thin-layer chromatography (TLC) on silica gel. TLC was performed on Silica Gel 60 F254 plates (Merck) with UV detection, or using a molybdate developing solution [5% aq H_2SO_4 with 4% $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ and 0.2% $\text{Ce}(\text{SO}_4)_2$] followed by heating at 120°C. Flash column chromatography was performed on silica gel 230–400 mesh (Merck). The petroleum ether used as eluent in chromatography has boiling range of 40–60°C. ^1H and ^{13}C -NMR spectra were recorded on a Varian 400 MHz Mercury instrument at 298 K. Chemical shifts are reported in ppm downfield from TMS as internal standard. Mass spectra were recorded on ESI-MS triple quadrupole (model API2000 QTrapTM, Applied Biosystems).

Phosphoryl 2-deoxy-3-*O*-tetradecanoyl-2-tetradecanoylamino- α -D-glucopyranoside

(1): Compound **8** (0.05 g, 0.05 mmol) was dissolved in dry $\text{CH}_2\text{Cl}_2/\text{MeOH}$ 1:2 (6 mL), then Pd on activated charcoal in catalytic amount and AcOH were added. The reaction was stirred at r.t. under H_2 atmosphere overnight, monitoring the disappearance of starting material by TLC (toluene/AcOEt 7:3). Triethylamine (100 μL) was then added to the reaction mixture, and the suspension was filtered on a syringe filter in order to remove Pd/C catalyst, and washed with CH_2Cl_2 . The product was then passed on a Amberlite IR 120 Na^+ exchange resin in order to remove triethylamine and give compound **1** as sodium salt (0.04 g, quantitative). ^1H NMR (400 MHz, $\text{CD}_3\text{OD} + 3\% \text{D}_2\text{O}$, 25 °C, TMS): δ = 5.44 (dd, $^3J(\text{H},\text{P})$ = 6.8 Hz, $^3J(\text{H},\text{H})$ = 3.4 Hz, 1H; H-1), 5.20 (dd, $^3J(\text{H},\text{H})$ = 10.6, 9.4 Hz, 1H; H-3), 4.15 (dt, $^3J(\text{H},\text{H})$ = 5.7, 3.2, 1H; H-2), 3.95 (m, 1H; H-5), 3.85 (dd, $^3J(\text{H},\text{H})$ = 11.8, 2.0 Hz, 1H; H-6a), 3.72 (dd, $^3J(\text{H},\text{H})$ = 11.8, 5.2 Hz, 1H; H-6b), 3.63–3.54 (m, 1H; H-4), 2.42–2.27 (m, 2H; $\text{CH}_2\alpha$ chains), 2.27–2.14 (m, 2H; $\text{CH}_2\alpha$ chains), 1.56 (m, 4H; $\text{CH}_2\beta$ chains), 1.37–1.20 (m, 40H; CH_2 bulk), 0.89 (t, $^3J(\text{H},\text{H})$ = 6.8 Hz, 6H; CH_3). ^{13}C NMR (101 MHz, $\text{CD}_3\text{OD} + 3\% \text{D}_2\text{O}$, 25 °C, TMS): δ = 175.23, 174.18, 93.77, 73.33, 72.91, 68.06, 60.60, 51.83, 35.80, 33.81, 33.31, 31.67, 29.47, 29.42, 29.38, 29.29, 29.19, 29.14, 29.08, 28.95, 25.69, 25.31, 24.60, 24.56, 22.35, 13.16, 7.88. ESI MS calc. 679.44, found 678.6, 339.4.

2-deoxy-4-O-phosphoryl-3-O-tetradecanoyl-2-tetradecanoylamino- α,β -D-

glucopyranose (2): Compound **11** (0.14 g, 0.15 mmol) was dissolved in dry $\text{CH}_2\text{Cl}_2/\text{MeOH}$ 1:2 (6 mL), then Pd on activated charcoal was added in catalytic amount. The reaction was stirred at r.t. under H_2 overnight, monitoring the disappearance of starting material by TLC (ETP/AcOEt 7:3). Triethylamine (100 μL) was added to the reaction mixture, and the suspension was filtered on a syringe filter in order to remove Pd/C catalyst. The pure triethylammonium salt was then passed on a Amberlite IR 120 Na^+ exchange resin in order to remove triethylamine and give compound **2** as sodium salt (mixture of α and β anomers, 5:1), in quantitative yield. ^1H NMR (400 MHz, $\text{CD}_3\text{OD} + 2.5\% \text{CDCl}_3$, 25 $^\circ\text{C}$, TMS): δ = 7.66 (d, $^3J(\text{H,H})$ = 9.6 Hz, 1H; NH), 5.34 (t, $^3J(\text{H,H})$ = 9.8 Hz, 1H; H-3 α), 5.14 (t, $^3J(\text{H,H})$ = 9.7 Hz, 1H; H-3 β), 5.05 (d, $^3J(\text{H,H})$ = 3.6 Hz, 1H; H-1 α), 4.68 (d, $^3J(\text{H,H})$ = 8.4 Hz, 1H; H-1 β), 4.26–4.10 (m, 3H; H-2 α , H-4 α , H-4 β), 3.98–3.90 (m, 3H; H-5 α , H-6 $\alpha\alpha$, H-6 $\alpha\beta$), 3.85–3.75 (m, 2H; H-2 β ; H-6 $\beta\beta$), 3.68 (m, 1H; H-6 $\beta\alpha$), 3.40 (m, 1H; H-5 β), 2.44–2.11 (m, 8H; $\text{CH}_2\alpha$ chains), 1.56 (m, 8H; $\text{CH}_2\beta$ chains), 1.45–1.17 (m, 80 H; CH_2 bulk), 0.89 (t, $^3J(\text{H,H})$ = 6.7 Hz, 12H; CH_3). ^{13}C NMR (101 MHz, $\text{CD}_3\text{OD} + 2.5\% \text{CDCl}_3$, 25 $^\circ\text{C}$, TMS): δ = 78.97, 178.15, 99.68, 95.74, 75.93, 75.44, 75.16, 64.65, 59.48, 56.55, 50.39, 46.21, 40.31, 39.87, 37.99, 35.90, 33.69, 33.63, 33.59, 33.55, 33.52, 33.33, 33.25, 29.98, 28.69, 26.55, 17.27, 11.93. ESI MS calc. 679.44, found 678.52.

Phosphoryl 2-deoxy-4-O-phosphoryl-3-O-tetradecanoyl-2-tetradecanoylamino- α -D-

glucopyranoside (3): Compound **13** (0.08 g, 0.06 mmol) was dissolved in dry $\text{CH}_2\text{Cl}_2/\text{MeOH}$ 1:2 (5 mL), then Pd on activated charcoal was added in catalytic amount. The reaction was stirred at r.t. under H_2 atmosphere overnight (TLC AcOEt). Triethylamine (150 μL) was then added to the reaction mixture and the suspension was filtered on a syringe filter. The triethylammonium salt was then passed on a Amberlite IR 120 Na^+ exchange resin in order to remove triethylamine and give compound **3** as sodium salt in quantitative yield (61 mg). ^1H NMR (400 MHz, CD_3OD , 25 $^\circ\text{C}$, TMS): δ = 5.45 (dd, $^3J(\text{H,P})$ = 6.4, $^3J(\text{H,H})$ = 3.5 Hz, 1H; H-1), 5.37 (t, $^3J(\text{H,H})$ = 9.9 Hz, 1H; H-3), 4.31 – 4.18 (m, 2H; H-2, H-4), 4.02 – 3.95 (m, 2H; H-5, H-6 α), 3.72 (d, $^2J(\text{H,H})$ = 12.6 Hz, 1H; H-6 β), 2.46 – 2.14 (m, 4H; $\text{CH}_2\alpha$ chains), 1.56 (m, 4H; $\text{CH}_2\beta$ chains), 1.39 – 1.20 (m, 40H, CH_2 chains), 0.89 (t, $^3J(\text{H,H})$ = 6.6 Hz, 6H; CH_3 chains). ^{13}C NMR (101 MHz, CD_3OD , 25 $^\circ\text{C}$, TMS): δ = 174.60, 173.29, 94.11, 72.22, 71.89, 70.86, 60.22, 53.41, 52.16, 46.14, 35.87, 33.77, 31.72, 29.51, 29.45, 29.42, 29.37, 29.35, 29.32, 29.28, 29.15, 29.09, 25.71, 24.87, 24.49, 22.37, 13.09, 7.74. ESI MS calc. 759.41, found 758.5, 378.7.

Biology

HEK-Blue™ assay—HEK-Blue™ hTLR4 cells (HEK-Blue™ LPS Detection Kit, InvivoGen) were cultured according to manufacturer's instructions. Cells were cultured in DMEM high-glucose medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 1 $\times$ Normocin™ (InvivoGen), 1 $\times$ HEK-Blue™ Selection (InvivoGen). Cells were detached by the use of a cell scraper and the cell concentration was estimated by using a cell counter. Cells were then diluted in DMEM high glucose medium supplemented with 10% FBS, 2 mM glutamine, 1 $\times$ Normocin™ (InvivoGen), and 200 μL of cell suspension (20000 or $2 \cdot 10^4$ cells) were added to each well. After overnight incubation (37 $^\circ\text{C}$, 5% CO_2 , 95% humidity), cells reached 80% confluency and supernatant was removed, cell monolayers

were washed with warm PBS without Ca^{2+} and Mg^{2+} , pre-incubated for 30 min in 190 μL DMEM + 0.03% BSA, supplemented with compounds **1**, **2** or **3** (different concentrations in different wells, from 0 to 50 μM). LPS (*E. coli* O55:B5 strain, *Sigma-Aldrich*) at a final concentration of 10, 100 or 1000 ng/mL was then added as stimulus (10 μL /well) and cells incubated overnight in the same conditions as above. After the incubation, supernatants were collected. 50 μL of each sample were added to 100 μL of a 0.8 mM pNPP solution in PBS. Plates were incubated in the dark at room temperature and then analyzed with a spectrophotometer (absorbance at 405 nm).

Murine bone marrow-derived macrophages (BMM Φ) assay—Murine BMM Φ were obtained from wild type and CD14^{-/-} mice and cultured following the published procedure.^[30] Cells were washed with PBS and detached using 2 mM EDTA in PBS. Cellular suspension was then centrifuged at 1000 rpm for 5 min and cellular pellet resuspended in DMEM high glucose + FBS 10% and plated in a 96-well plate (20000 or $2 \cdot 10^4$ cells/well). After overnight incubation (37 °C, 5% CO₂, 95% humidity), supernatant was removed, cell monolayers were washed with warm PBS without Ca^{2+} and Mg^{2+} , pre-incubated for 30 min in 190 μL DMEM + 0.03% BSA, supplemented with compounds **1**, **2** or **3** (different concentrations in different wells, from 0 to 50 μM). LPS (*E. coli* O55:B5 strain, *Sigma-Aldrich*) at a final concentration of 10, 100 or 1000 ng/mL was then added as stimulus (10 μL /well) and cells incubated overnight in the same conditions as above. After the incubation, supernatants were collected and TNF concentration was detected through an ELISA.

Flow cytometry analysis

BMM Φ were washed twice with PBS and detached using 2 mM EDTA in PBS. Cellular suspension was then centrifuged at 1000 rpm for 5 min and cellular pellet resuspended in DMEM high glucose + FBS 10% (100 μL) and incubated at 37 °C, 5% CO₂, and 95% humidity for 40 or 70 min with compound **1**, **2**, **3** (10 μM) or LPS (1 $\mu\text{g}/\text{mL}$). Cellular suspensions were then centrifuged at 1000 rpm, 4°C, for 5 min and cellular pellet resuspended with ice-cold PBS. The receptor-specific fluorescent antibodies were then added (aCD14: FITC Rat anti-mouse CD14, clone SA2-8, *eBioscience*; aTLR4: PE Rat anti-mouse TLR4, clone SA15-21, *Biolegend*) 1 $\mu\text{g}/\text{mL}$, for 20 min in ice in the dark. The cells were then washed two times with ice-cold PBS and fluorescence was analyzed with a FaCSCalibur flow cytometer (*BD Biosciences*).

Production and isolation of MD-2

Recombinant MD-2 was produced in *Escherichia coli* as described previously^[31] using solubilization of inclusion bodies in 6M GdnHCl followed by purification and refolding on a C8 reversed phase column chromatography. Eluted fractions were lyophilized and dialyzed against the Milli-Q water. Biological activity of each batch of MD-2 for the ability to support LPS induced TLR4 activation in HEK293 cells transfected with TLR4.

Determination of CMC via Pyrene Fluorescence Measurements^[29]

For every sample, independently of the synthetic compounds concentration, a final concentration 0.6 μM on pyrene is desired. A mother solution 1 mM of pyrene in THF is

prepared in a 25 mL volumetric flask, and diluted into a 237 mM solution (6 mL of the concentrated solution, followed by addition of 19 mL of THF, to a final volume of 25 mL). 252 μ L of this solution is then diluted with Milli-Q water, to a final volume of 100 mL. Aqueous solutions of each compound (0.6 μ M in pyrene) were prepared at concentrations from 1 mM to 0.05 μ M, by two-fold serial dilutions. The 1 mM solutions were prepared by adding 0.6 μ M pyrene solution to each compound and sonicating for 1 h. The serial dilutions were incubated for 1 h at 37.0 °C. For fluorescence measurements, 1.5 mL of solution was placed in conventional 1-cm quartz cuvettes; fluorescence spectra were recorded with an Varian Cary Eclipse spectrofluorophotometer at 37 ± 0.1 °C, using 5 mm excitation and emission slits. The onset of micelle formation can be observed in a shift of the fluorescence excitation spectra of the samples at an emission wavelength of 372 nm. In the concentration range of aqueous micellar solutions, a shift of the excitation band in the 335 nm region toward higher wavelengths confirms the incorporation of pyrene in the hydrophobic interior of micelles. The ratio of the fluorescence intensities at 339 and 335 nm was used to quantify the shift of the broad excitation band. The critical micelle concentrations were determined from the crossover point in the low concentration range.

NMR binding experiments

NMR experiments were performed on a 600 MHz DRX spectrometer (Bruker) equipped with a cryo-probe. ^1H -NMR spectra were recorded at 298 K, by acquiring 120 scans. DOSY spectra were recorded at 311 K with the `steppedgs19` Bruker pulse sequence by acquiring 160 scans, with diffusion time of 300ms, a gradient length of 1.9 ms, and a gradient ramp from 2% to 95% in 32 linear steps. Protein samples were prepared diluting the stock solution of MD-2 (0.11 mM in deuterated acetate buffer at pH=5) with the same buffer. Ligand samples were prepared dissolving solid compound **3** in deuterated acetate buffer at pH=5. For ^1H -NMR experiments the final concentrations reached for the analyzed samples were: 30 μ M MD-2 and 150 μ M compound **3**. While for DOSY experiments final concentrations of 60 μ M MD-2 and 300 μ M compound **3** were needed.

Molecular modeling

3D coordinates of compound **3** were built by means of Maestro (version 9.3, Schrödinger, LLC, New York, NY, 2012). Phosphate groups were considered as monoprotonated. Molecular mechanics optimization (UFF force field), semi-empirical calculations (AM1), and DFT (B3LYP/6-31G*) were subsequently applied using Gaussian03.^[32] Final geometry was submitted to MD simulations with implicit water and MM3* as force field, by using Schrödinger Maestro 9.3 Impact 5.8, [Maestro, version 9.3, Schrödinger, LLC, New York, NY, 2012. Suite 2012: Impact version 5.8, Schrödinger, LLC, New York, NY, 2012] and the MM3* force field, dielectric constant: 80.0 number of MD steps 100, and time step of 0,001 ps. 3D coordinates of human MD-2 protein (from PDB 2E59), and CD14 protein (from 1WWL) were refined and minimized under the Protein Preparation Wizard module of Maestro, using amber force field.^[33] In the case of CD14, only sequence ranging from Ala3 to Leu130 was considered for docking purposes. Compound **3** was docked into both MD-2 and CD14 proteins using AutoDock 4.2.^[25], and separately using AutoDock Vina 1.1.2.^[26] Predicted binding energies ranged from -2 to -6 kcal/mol in AutoDock results, and from -6 to -9 kcal/mol in AutoDock Vina results. For MD-2 the Autogrid grid point spacing was set

at 0.375 Å, center coordinates of the grid box were -0.379, 17.201, 16.216 (x, y, z), and number of grid points in xyz was 58, 92, 82. For CD14 the Autogrid grid point spacing was set at 0.375 Å, center coordinates of the grid box were 13.500, 51.000, 56.500 (x, y, z), leading to 66, 72, 88 (x, y, z) grid points. All allowed torsional bonds were considered rotatable. Docking calculations with AutoDock were performed using Genetic Algorithm (number of runs 250, number of individuals in population 150). Docking calculations with AutoDock Vina were also performed. Coordinates and dimensions of grid boxes, starting geometries and general methodology were the same as for AutoDock. 3D structures of the docked complexes were optimized by performing MD simulations with Impact (implicit water, and AMBER* force field).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Julio Rodriguez Lavado for experimental CMC determination. We thank the COST action BM 1003 “Microbial cell surface determinants of virulence as targets for new therapeutics for Cystic Fibrosis”; FP thanks the US National Institutes of Health (NIH)/National Institute of Allergy and Infectious Diseases (NIAID), project: “Regulation of MD2 function and expression” (1R01AI059372), the COST Action CM1102 “Multivalent Glycosystems for Nanoscience -MultiGlycoNano”; the Italian Ministry of University and Research (MIUR), PRIN 2010-11, project: “Italian network for the development of multivalent nanosystems”. Spanish MINECO (Grants CTQ2012-32025, and CTQ2011-22724), Universidad CEU San Pablo (PC14/2011 and PC13/2012), as well as the European Commission granted GLYCOPHARM ITN-project are also gratefully acknowledged. E. K. thanks Airbus-Military for a research contract. The Associazione Italiana per la Ricerca sul Cancro (AIRC), the Fondazione Cariplo (Grant 2010-0678) and Regione Lombardia are also gratefully acknowledged.

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Figure 1. HEK-blue[™] cells assay: Compound **3** inhibits in a dose-dependent way the LPS-triggered TLR4 activation (monitored as sAP colorimetric reaction, normalized data, n=3 experiments). Low concentrations of LPS (10 ng/mL) gave an IC_{50} value for compound **3** of 0.46 μM and high doses of LPS (1 $\mu\text{g/mL}$) shifted the IC_{50} to 3.42 μM .

Figure 2.

Effect of compounds **1–3** on LPS-induced TNF- α production by BM-derived macrophages. WT or CD14 $^{-/-}$ BM-macrophages were pre-incubated for 30 min with synthetic compounds and then treated with high LPS concentration (1 μ g/mL, black squares) or low LPS (10 ng/mL, open circles). Readout was the TNF- α production after one night incubation.

Figure 3.

CD14 and TLR4 internalization induced by compounds 1, 2 and 3. BMM Φ were treated with LPS (1 μ g/mL) or with synthetic compounds and incubated at 37 °C for the time indicated. Flow cytometry was then used to examine receptor endocytosis by determining the surface levels of the proteins indicated. Panels represent the mean fluorescence intensity (MFI) of specific receptor staining at each time point.

Figure 4.

A) ¹H-NMR of 30 μM MD-2 protein in deuterated acetate buffer at pH=5, 298K, 120 scans; B) ¹H-NMR of 30 μM MD-2 protein and 150 μM compound 3 in deuterated acetate buffer at pH=5, 298 K, 120 scans; C) ¹H-NMR of 150 μM compound 3 in deuterated acetate buffer at pH=5, 298 K, 120 scans.

Figure 5.

AutoDock binding poses of compound 3 characterized for having two (green) or one (magenta) FA chain oriented inside the lipophilic MD-2 pocket. Lipid IVa is shown as reference in CPK colours.

Scheme 1.

E. coli lipid A and its biosynthetic precursor lipid X, mono- (**1** and **2**) and diphosphorylated (**3**) lipid X mimetics.

Scheme 2.

Reagents and conditions: a) PPh_3 , THF/ H_2O , 60 °C, 87%; b) myristic acid, EDC, DMAP, CH_2Cl_2 , 97%; c) TBAF, AcOH, THF -15°C to RT, 76%; d) tetrabenzyl diphosphate, $\text{LiN}(\text{TMS})_2$, dry THF, -78 to -20°C , 43%; e) H_2 , Pd/C, AcOH, dry MeOH/ CH_2Cl_2 , quant.; f) NaCNBH_3 , 4Å MS, dry THF, 84%; g) $(\text{BnO})_2\text{PNi-Pr}_2$, imidazolium triflate, dry CH_2Cl_2 , then *m*-CPBA, 0 °C, 42%; h) TBAF, AcOH, THF, -15°C to RT, 57%; i) H_2 , Pd/C, AcOH, dry MeOH/ CH_2Cl_2 , quant.; j) tetrabenzyl diphosphate, $\text{LiN}(\text{TMS})_2$, dry THF, -78 to -20°C , 71%; k) H_2 , Pd/C, AcOH, dry MeOH/ CH_2Cl_2 , quant.; l) NaCNBH_3 , 4Å MS, dry THF, then HCl in dioxane until pH=1.5, 61%; m) $(\text{BnO})_2\text{PNi-Pr}_2$, imidazolium tosylate, dry CH_2Cl_2 , then *m*-CPBA, 0 °C, 38%.