

Evidence of a Specific Interaction between New Synthetic Antisepsis Agents and CD14[†]

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ABSTRACT: Synthetic molecules derived from natural sugars with a positively charged amino group or ammonium salt and two lipophilic chains have been shown to inhibit TLR4 activation in vitro and in vivo. To characterize the mechanism of action of this class of molecules, we investigated possible interactions with the extracellular components that bind and shuttle endotoxin [lipopolysaccharide (LPS)] to TLR4, namely, LBP, CD14, and MD-2. Molecules that inhibited TLR4 activation inhibited LBP·CD14-dependent transfer of endotoxin monomers derived from aggregates of tritiated lipooligosaccharide (³H]LOS) from *Neisseria meningitidis* to MD-2·TLR4, resulting in a reduced level of formation of a (³H]LOS·MD-2·TLR4_{ECD})₂ (*M_r* ~ 190000) complex. This effect was due to inhibition of the transfer of [³H]LOS from aggregates in solution to sCD14 with little or no effect on [³H]LOS shuttling from [³H]LOS·sCD14 to MD-2. These compounds also inhibited transfer of the [³H]LOS monomer from full-length CD14 to a truncated, polyhistidine-tagged CD14. Dose-dependent inhibition of the transfer of [³H]LOS between the two forms of CD14 was observed with each of three different synthetic compounds that inhibited TLR4 activation but not by another structurally related analogue that lacked TLR4 antagonistic activity. Saturation transfer difference (STD) NMR data showed direct binding to CD14 by the synthetic TLR4 antagonist mediated principally through the lipid chains of the synthetic compound. Taken together, our findings strongly suggest that these compounds inhibit TLR4 activation by endotoxin by competitively occupying CD14 and thereby reducing the level of delivery of activating endotoxin to MD-2·TLR4.

Innate immunity is the first line of defense against microbial infections. Defense responses are activated when microbial components are recognized by a variety of pathogen sensors, including Toll-like receptors (TLRs) that activate the host defense effector system by rapidly triggering pro-inflammatory processes (1). Among microbial components, lipopolysaccharides (LPS)¹ and lipooligosaccharides (LOS) and their bioactive portions, the lipodisaccharide lipid A, commonly defined as endotoxins (E), are potent stimulants of immune responses, but small differences in LPS structure can have a great influence on host immune responses (2). Endotoxin is an amphiphilic molecule and under physiological conditions is an integral membrane constituent. After extraction and purification, endotoxin forms large aggregates whose supramolecular structure depends on the chemical structure of endotoxin and, in particular, the lipid A moiety (3–5). However, as for every amphiphilic system, monomers are also present in a dynamic equilibrium. The

induction of inflammatory responses by endotoxin is achieved by the coordinate and sequential action of four principal endotoxin binding proteins: the lipopolysaccharide binding protein (LBP), the cluster differentiation antigen CD14, the myeloid differentiation protein (MD-2), and Toll-like receptor 4 (TLR4) (6). LBP interacts with endotoxin-rich bacterial membranes and purified endotoxin aggregates, catalyzing extraction and transfer of E monomers to CD14 in the presence of serum albumin (7, 8). Monomeric E·CD14 complexes are the most efficient vehicle for transfer of E monomers to MD-2 and to the MD-2·TLR4 heterodimer, explaining the importance of LBP and CD14 for LPS signaling at low endotoxin concentrations (9, 10). CD14 also has an important role in TRIF-dependent intracellular signaling triggered after TLR4 activation by endotoxin (11). The transfer of LPS from CD14 to MD-2, coupled with binding of MD-2 to TLR4, is required for TLR4 activation (12–14). Activation includes the formation of a dimer of the ternary (TLR4·MD-2·E)₂ complex (15). Receptor dimerization leads to the recruitment of adapter proteins to the intracellular domain of TLR4, initiating the intracellular signal cascade that culminates in translocation of transcription factors to the nucleus and the biosynthesis of cytokines. The very recent determination of the crystal structure of the (TLR4·MD-2·LPS)₂ complex (16), together with crystallographic data for MD-2 bound to TLR4 antagonists lipid IVa (17) and Eritoran (18), has revealed some fundamental structural aspects of the TLR4 dimerization process and the molecular basis of TLR4 agonism and antagonism. The majority

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¹Abbreviations: CD14, cluster differentiation antigen; tCD14, truncated CD14; sCD14, soluble CD14; DMSO, dimethyl sulfoxide; E, endotoxin; FPLC, fast protein liquid chromatography; HEK, human embryonic kidney; HSA, human serum albumin; LPS, lipopolysaccharide; LBP, lipid binding protein; LOS, lipooligosaccharide; MD-2, myeloid differentiation protein; *M_r*, apparent molecular weight; pBAC3, bacterial artificial chromosome (BAC) vector; STD, saturation transfer difference; TLR4, Toll-like receptor 4; TLR4_{ECD}, TLR4 ectodomain.

of antiseptics agents designed to be TLR4 antagonists, such as Eritoran (19), are comprised of a $\beta(1-6)$ *N*-acetylglucosamine disaccharide scaffold with two phosphates at positions 1 and 4' and four lipid chains instead of the six present in lipid A (20–23). Comparison of the crystal structure of the (TLR4·MD-2·LPS)₂ complex (16) and that of the TLR4·MD-2·Eritoran complex (18) indicates that the size of the MD-2 pocket is the same whether hexaacylated agonists or tetraacylated antagonists are bound. The MD-2 cavity volume can readily accommodate the four lipid chains of antagonists, and additional space for lipid binding is generated, at least in the case of hexaacylated *Escherichia coli* LPS₅ by displacing the glucosamine backbone upward by ~5 Å (16). This shift of the anomeric phosphate and resulting rearrangement of the lipid A acyl chains may be essential for the interaction of activating LPS·MD-2 from one TLR4·MD-2·LPS ternary complex with TLR4 from a second ternary complex, leading to formation of the (TLR4·MD-2·LPS)₂ dimer.

Many other compounds whose structures are not related to that of lipid A have also been described that interfere with TLR4 activation. These include the cyclohexene derivative named TAK242 (24, 25), now in clinical phase III trials, and both synthetic and natural (host) polycationic amphiphiles that, by binding LPS, sequester LPS from the CD14/MD-2/TLR4 pathway and protect animals against endotoxin-induced lethality (26–28). We recently developed a new class of inhibitory compounds, namely, amphiphilic glycolipids **1** and **2** and benzylammonium lipid **3** (Figure 1). We found that these compounds (**1**–**3**) inhibit LPS-induced TLR4 activation on HEK/TLR4 cells and LPS-induced septic shock in mice (29, 30). Compounds **1** and **2** are also able to inhibit other pathologies caused by TLR4 activation, such as inflammation and neuropathic pain (31). In contrast, glycolipid **4**, which differs in structure from compounds **1** and **2** only by the presence of a neutral methoxyamino group instead of a charged amine, was inactive both in vitro and in vivo. The aim of this work was to clarify the mode of action of these molecules with a particular focus on the lead compound molecule **1**, by analyzing possible interactions with the extracellular components that bind and shuttle endotoxin to TLR4, namely, LBP, CD14, and MD-2 (free and TLR4-bound).

EXPERIMENTAL PROCEDURES

Materials. [³H]LOS (25000 cpm/pmol) was metabolically labeled and isolated from an acetate auxotroph of *Neisseria meningitidis* serogroup B as described previously (32). LBP and sCD14 were gifts from Xoma (Berkley, CA) and Amgen Corp. (Thousand Oaks, CA), respectively. Human serum albumin (HSA) was obtained as an endotoxin-free, 25% stock solution (Baxter Health Care, Glendale, CA). Chromatography matrices (Sephacryl HR S200 and S300) were purchased from GE Healthcare, and the silica-based metal chelation matrix, HisLink, was from Promega. ESF921 medium for High Five insect cells was purchased from Expressions Systems. Molecules **1**–**4** (Figure 1) were prepared, purified, and characterized as previously described (29, 30). In the assays described below, stocks of molecules **1**–**4** (~15 mM) were freshly prepared in a 50% DMSO/50% ethanol mixture (v/v) and then further diluted with a PBS/0.1% HSA mixture to the desired concentration.

Preparation of Recombinant Proteins. Preparative amounts of sMD-2 and a truncated form of CD14 were generated

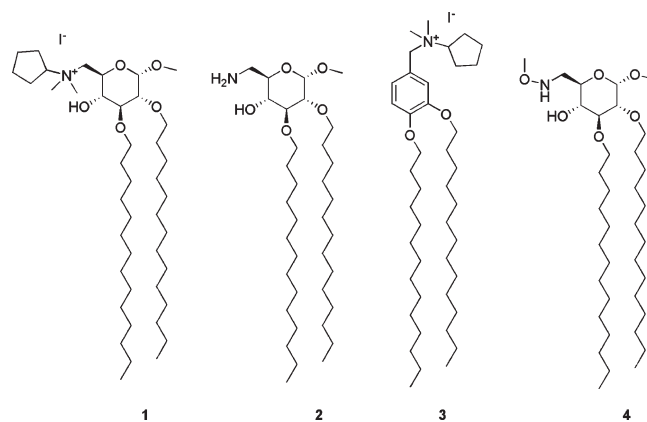


FIGURE 1: Structures of methyl 6-deoxy-6-*N*-dimethyl-*N*-cyclopentylammonium-2,3-di-*O*-tetradecyl- α -D-glucopyranoside (**1**), methyl 6-deoxy-6-amino-2,3-di-*O*-tetradecyl- α -D-glucopyranoside (**2**), *N*-(3,4-bis(tetradecyloxy)benzyl)-*N*-cyclopentyl-*N,N*-dimethylammonium (**3**), and methyl 6-deoxy-6-methoxyamino-2,3-di-*O*-tetradecyl- α -D-glucopyranoside (**4**).

from infections of High Five (Invitrogen) insect cells with baculovirus containing the cDNA for human MD-2 inserted into pBAC3 (His₆-MD-2) or for human tCD14 (amino acids 1–156, tCD14-His₆) inserted into pBAC11 as described previously (15). Conditioned medium containing MD-2 was stored at –80 °C until it was needed. tCD14-His₆ was purified using Ni FF Sepharose resin (GE Healthcare) on an Explorer100 fast protein liquid chromatography (FPLC) system (GE Healthcare) with an imidazole gradient. Purified tCD14-His₆ was stored at 4 °C (15). Conditioned medium containing MD-2 associated with the TLR4 ectodomain (TLR4_{ECD}), MD-2/TLR4_{ECD}, was produced by transient transfection in HEK293T cells as described previously (15). Expression vectors containing the cDNA of interest for production of FLAG-TLR4_{ECD}, amino acids 24–631, (pFLAG-CMV-TLR4), and MD-2-FLAG-His (pEF-BOS) have been previously described and characterized (15). Media containing secreted proteins were concentrated 10–20-fold using a Millipore Centricon-10 before use. Conditioned medium containing secreted MD-2/TLR4_{ECD} proteins maintained activity to react with [³H]LOS·sCD14 for at least 6 months when stored at 4 °C.

Size Exclusion Chromatography. Sephacryl S300 and S200 columns were used for resolution and identification of different [³H]LOS·protein products [i.e., ([³H]LOS·MD-2·TLR4_{ECD})₂ (*M_r* ~ 190000), [³H]LOS·sCD14 (*M_r* ~ 60000), and [³H]LOS·MD-2 (*M_r* ~ 25000)]. For determination of apparent *M_r* values, these columns were calibrated with the following *M_r* standards: blue dextran (2 × 10⁶, *V*₀), thyroglobulin (650000), ferritin (440000), catalase (232000), IgG (158000), HSA (66000), ovalbumin (44500), myoglobin (17500), and vitamin B₁₂ (1200, *V*_i).

In experiments in which the formation of [³H]LOS·MD-2 and [³H]LOS·CD14 complexes was monitored, chromatographic analyses were conducted using a Sephacryl HR S200 column (1.6 cm × 30 cm) pre-equilibrated in PBS (pH 7.4) and 0.1% HSA and eluted in the same buffer at a flow rate of 0.5 mL/min at room temperature using AKTA Purifier or Explorer 100 fast protein liquid chromatography (GE Healthcare). Fractions of 1 mL were collected. In experiments in which the generation of (LOS·MD-2·TLR4_{ECD})₂ was also monitored, the reaction mixtures were applied to a Sephacryl HR S300 column (1.6 cm × 70 cm) and chromatography was conducted as described above.

Radioactivity ($[^3\text{H}]\text{LOS}$) in collected fractions was analyzed by liquid scintillation spectroscopy (Beckman LS liquid scintillation counter). The total recovery of $[^3\text{H}]\text{LOS}$ was $\geq 70\%$ in all experiments; experimental results in chromatograms are reported as the percent counts per minute of total radiolabeled LOS recovered.

Production and Purification of $[^3\text{H}]\text{LOS}_{\text{agg}}$ and $[^3\text{H}]\text{LOS}\cdot\text{sCD14}$ and $[^3\text{H}]\text{LOS}\cdot\text{MD-2}$ Complexes. $[^3\text{H}]\text{LOS}_{\text{agg}}$ and the $[^3\text{H}]\text{LOS}\cdot\text{sCD14}$ complex were prepared as previously described (8). Briefly, $[^3\text{H}]\text{LOS}_{\text{agg}}$ ($M_r > 20 \times 10^6$) were obtained after hot phenol extraction of $[^3\text{H}]\text{LOS}$ from metabolically labeled bacteria, followed by ethanol precipitation of $[^3\text{H}]\text{LOS}_{\text{agg}}$, and ultracentrifugation. Monomeric $[^3\text{H}]\text{LOS}\cdot\text{CD14}$ complexes ($M_r \sim 60000$) were prepared by treatment of $[^3\text{H}]\text{LOS}_{\text{agg}}$ for 30 min at 37°C with a substoichiometric amount of LBP (200:1 LOS:LBP molar ratio) and a 1–1.5-fold molar excess sCD14 followed by gel exclusion chromatography (Sephacryl S200, 1.6 cm \times 70 cm column) in PBS (pH 7.4) and 0.1% HSA to isolate the monomeric $[^3\text{H}]\text{LOS}\cdot\text{sCD14}$ complex. The $[^3\text{H}]\text{LOS}\cdot\text{MD-2}$ complex ($M_r \sim 25000$) was generated by treatment of the $[^3\text{H}]\text{LOS}\cdot\text{sCD14}$ complex (30 min at 37°C) with High Five insect cell medium containing His₆-MD-2 followed by isolation of the $[^3\text{H}]\text{LOS}\cdot\text{MD-2}$ complex by S200 chromatography. The radiochemical purity of the $[^3\text{H}]\text{LOS}\cdot\text{sCD14}$ and $[^3\text{H}]\text{LOS}\cdot\text{MD-2}$ complexes was confirmed by S200 chromatography (12).

Effect of Synthetic Molecules on the Formation of the $([^3\text{H}]\text{LOS}\cdot\text{MD-2}\cdot\text{TLR4}_{\text{ECD}})_2$ Complex from $[^3\text{H}]\text{LOS}_{\text{agg}}$. In these experiments, sCD14 (0.8 nM, final concentration) was preincubated with LBP (4 pM, final concentration) with or without synthetic molecules (0–10 μM) for 30 min at 37°C in 0.875 mL of PBS (pH 7.4) and 0.1% HSA. After this preincubation, $[^3\text{H}]\text{LOS}_{\text{agg}}$ was added (up to 5 μL , yielding a final LOS concentration of 0.8 nM) and the mixture was incubated for an additional 30 min at 37°C . This was followed by addition of conditioned HEK293T cell medium containing a preformed MD-2·TLR4_{ECD} heterodimer (15) (0.125 mL, ca. 0.2 nM, final concentration, of reactive MD-2·TLR4_{ECD} heterodimer) and an additional incubation for 15 min at 37°C . Analysis of the reaction products was conducted by gel size exclusion chromatography as described above.

Effect of the Synthetic Molecules on the Transfer of LOS from the $[^3\text{H}]\text{LOS}\cdot\text{sCD14}$ Complex to either His-Tagged tCD14 or sMD-2. Conditioned medium containing either His-tagged protein (corresponding to a final concentration in the incubation mixture of ~ 1.2 nM) was preincubated with sCD14 (0.8 nM) with or without synthetic molecules in PBS and 1% HSA for 30 min at 37°C . sCD14 was added to the preincubation to facilitate interaction of the synthetic compound with either His-tagged protein. LBP was not needed. This preincubation was followed by incubation for 30 min at 37°C with the $[^3\text{H}]\text{LOS}\cdot\text{sCD14}$ complex (0.8 nM) to allow transfer of $[^3\text{H}]\text{LOS}$ to available His-tagged tCD14 or MD-2. Products of the reactions were analyzed by size exclusion chromatography (see above) and/or by cocapture with HISLINK resin (see below).

Evaluation of the Transfer of $[^3\text{H}]\text{LOS}$ to His₆-Tagged Proteins by Cocapture with Metal Chelating Resin. tCD14-His₆ or His₆-sMD-2 (1.6 nM, final concentration) was preincubated in 0.3 mL of PBS and 1% HSA with or without LBP (4 pM) for 30 min at 37°C with or without different concentrations of synthetic compounds. $[^3\text{H}]\text{LOS}_{\text{agg}}$ (0.8 nM) (with tCD14)

or the $[^3\text{H}]\text{LOS}\cdot\text{sCD14}$ complex (0.8 nM) (with tCD14 or with MD-2) was added followed by another incubation for 30 min at 37°C . The reaction mixture was then incubated with HISLINK resin (20 μL) for 15 min at 25°C , allowing His-tagged tCD14 and sMD-2 to be adsorbed onto the beads. The resin was spun down for 2 min at 2000g; the supernatant was removed, and the resin was washed twice with PBS and 1% HSA using the same procedure. The $[^3\text{H}]\text{LOS}$ absorbed onto the beads or recovered in the supernatant was quantified by liquid scintillation spectroscopy.

Endotoxin Displacement Assay. tCD14-His₆ (10 μL , final concentration of 1.6 nM) was preincubated in PBS (pH 7.4) and 0.1% HSA in the presence of LBP (8 pM) and $[^3\text{H}]\text{LOS}_{\text{agg}}$ (1.6 nM) for 30 min at 37°C . These samples were then incubated for an additional 30 min at 37°C with or without synthetic compounds (10 μM), to allow displacement of bound $[^3\text{H}]\text{LOS}$ by the synthetic compound. Accumulation of the $[^3\text{H}]\text{LOS}\cdot\text{His}_6$ -tCD14 complex was monitored by size exclusion chromatography and by capture by HISLINK resin as described above.

Assay of Glycolipid Molecule 1–sCD14 Interaction by Saturation Transfer Difference (STD) Nuclear Magnetic Resonance (NMR) Spectroscopy. A freshly prepared stock (1.5 mM) of molecule 1 [in a 1:1 (v/v) DMSO-*d*₆/methanol-*d*₄ mixture] was diluted to 19 μM in a buffer containing 10 mM sodium phosphate (pH 6.8) in 100% ²H₂O (sample A). An aliquot of sample A (0.5 mL) was mixed with 2 μL of a 120 μM stock solution of sCD14 (in the same sodium phosphate buffer) to give a final sCD14 concentration of 0.5 μM (sample B). This sample was used to collect the STD NMR data. The control sample (sample C) contained 0.5 μM sCD14 alone in the same buffer [10 mM sodium phosphate (pH 6.8) in 100% ²H₂O].

STD NMR data were collected at 25°C on a Bruker Avance II 800 MHz NMR spectrometer equipped with a sensitive cryoprobe. STD data were collected as previously described (33–36). Since no aromatic resonances are present for molecule 1, the sCD14 protein can be selectively saturated by irradiation (on-resonance) at 7.6 and 8.0 ppm on the protein resonances with a train of Gauss-shaped pulses with a total saturation time of 1.0 s. The off-resonance radiation was performed at a chemical shift of 13.0 ppm where no resonances were observed. The on-resonance and off-resonance spectra were recorded in an interleaved fashion. The total number of scans collected was 10K. The spectra were processed with topspin version 2.1 on the Bruker spectrometer.

RESULTS AND DISCUSSION

Molecule 1 Inhibits LBP/sCD14-Dependent Extraction and Transfer of $[^3\text{H}]\text{LOS}$ Monomers from $[^3\text{H}]\text{LOS}$ Aggregates to CD14 and to the MD-2·TLR4 Complex. Molecule 1 was selected as a lead compound for biochemical characterization. We have previously shown that this compound inhibits LPS- and lipid A-stimulated cytokine production in cellular and animal models (29, 30). To better define the mechanism of inhibitory action of molecule 1 on LPS (lipid A)-triggered TLR4 activation, we tested the ability of this compound to inhibit LBP/CD14-dependent transfer of endotoxin ($[^3\text{H}]\text{LOS}$) monomers from $[^3\text{H}]\text{LOS}$ aggregates to MD-2/TLR4, resulting in a reduced level of formation of a $([^3\text{H}]\text{LOS}\cdot\text{MD-2}\cdot\text{TLR4}_{\text{ECD}})_2$ complex ($M_r \sim 190000$) (15).

For this purpose, sCD14 was preincubated with LBP in the presence or absence of molecule 1 (10 μM) and then incubated

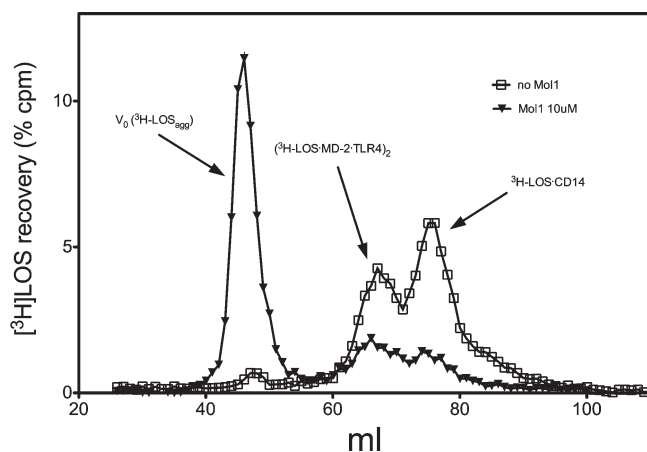


FIGURE 2: Molecule **1** inhibits conversion of $[^3\text{H}]\text{LOS}_{\text{agg}}$ to $[^3\text{H}]\text{LOS}\cdot\text{sCD14}$ and $([^3\text{H}]\text{LOS}\cdot\text{MD-2}\cdot\text{TLR4})_2$. sCD14 (0.8 nM) was preincubated with or without molecule **1** (10 μM) for 30 min at 37 °C in the presence of LBP (4 pM). Subsequently, $[^3\text{H}]\text{LOS}_{\text{agg}}$ (0.8 nM) was added, and this mixture was incubated for 30 min at 37 °C. After the second incubation, conditioned HEK293T cell medium containing preformed reactive MD-2·TLR4_{ECD} heterodimer (~0.2 nM, final concentration) was added and the mixture incubated again for 15 min at 37 °C. This reaction mixture was applied to Sephacryl S200 as described in Experimental Procedures used to measure conversion of $[^3\text{H}]\text{LOS}_{\text{agg}}$ (void volume) to $[^3\text{H}]\text{LOS}\cdot\text{sCD14}$ ($M_r \sim 60000$) and $([^3\text{H}]\text{LOS}\cdot\text{MD-2}\cdot\text{TLR4})_2$ ($M_r \sim 190000$). The chromatographic profiles shown are representative of at least four experiments. Overall recoveries of $[^3\text{H}]\text{LOS}$ were $\geq 70\%$.

with $[^3\text{H}]\text{LOS}_{\text{agg}}$ followed by conditioned medium containing a preformed MD-2·TLR4_{ECD} heterodimer. Size exclusion chromatography of the reaction mixture (Figure 2) showed that, in the absence of molecule **1**, virtually all $[^3\text{H}]\text{LOS}$ aggregates (LOS_{agg}) were converted to later-eluting species (i.e., smaller $[^3\text{H}]\text{LOS}$ -containing complexes) corresponding to $[^3\text{H}]\text{LOS}\cdot\text{sCD14}$ ($M_r \sim 60000$) and $([^3\text{H}]\text{LOS}\cdot\text{MD-2}\cdot\text{TLR4}_{\text{ECD}})_2$ ($M_r \sim 190000$). The identity of these complexes was confirmed by cocapture of $[^3\text{H}]\text{LOS}$ with immobilized monoclonal antibodies to CD14 (8) or to epitope tags in His₆-MD-2 or in Flag-TLR4_{ECD} (15) and by coelution with purified, defined $[^3\text{H}]\text{LOS}$ -protein complexes. The generation of the $[^3\text{H}]\text{LOS}\cdot\text{sCD14}$ complex reflects extraction and transfer of $[^3\text{H}]\text{LOS}$ monomers from $[^3\text{H}]\text{LOS}_{\text{agg}}$ to sCD14 by the combined action of LBP and sCD14. Formation of the $([^3\text{H}]\text{LOS}\cdot\text{MD-2}\cdot\text{TLR4}_{\text{ECD}})_2$ complex reflects transfer of $[^3\text{H}]\text{LOS}$ monomers from $[^3\text{H}]\text{LOS}\cdot\text{sCD14}$ to MD-2·TLR4_{ECD} (15). Thus, the chromatographic profile of $[^3\text{H}]\text{LOS}_{\text{agg}}$ incubated first with LBP and sCD14 and then with conditioned medium containing sMD-2·TLR4_{ECD} suggests that, under these experimental conditions, there is nearly complete extraction and transfer of $[^3\text{H}]\text{LOS}$ monomers from $[^3\text{H}]\text{LOS}_{\text{agg}}$ to $[^3\text{H}]\text{LOS}\cdot\text{sCD14}$ followed by transfer of $[^3\text{H}]\text{LOS}$ monomers from approximately half of the $[^3\text{H}]\text{LOS}\cdot\text{sCD14}$ complex formed to MD-2·TLR4_{ECD}. In contrast, in the presence of 10 μM molecule **1**, the extent of accumulation of both $[^3\text{H}]\text{LOS}\cdot\text{sCD14}$ and $([^3\text{H}]\text{LOS}\cdot\text{MD-2}\cdot\text{TLR4}_{\text{ECD}})_2$ was markedly reduced and most $[^3\text{H}]\text{LOS}$ was recovered in the void volume presumably as large $[^3\text{H}]\text{LOS}$ aggregates (Figure 2). The inhibition of accumulation of $[^3\text{H}]\text{LOS}\cdot\text{sCD14}$ suggested a primary effect of molecule **1** on LBP/sCD14-dependent extraction and transfer of $[^3\text{H}]\text{LOS}$ monomers from $[^3\text{H}]\text{LOS}_{\text{agg}}$ to $[^3\text{H}]\text{LOS}\cdot\text{sCD14}$.

To test this hypothesis more directly, we examined the effect of increasing concentrations of molecule **1** on LBP/sCD14-dependent conversion of $[^3\text{H}]\text{LOS}_{\text{agg}}$ to $[^3\text{H}]\text{LOS}\cdot\text{sCD14}$. As shown in

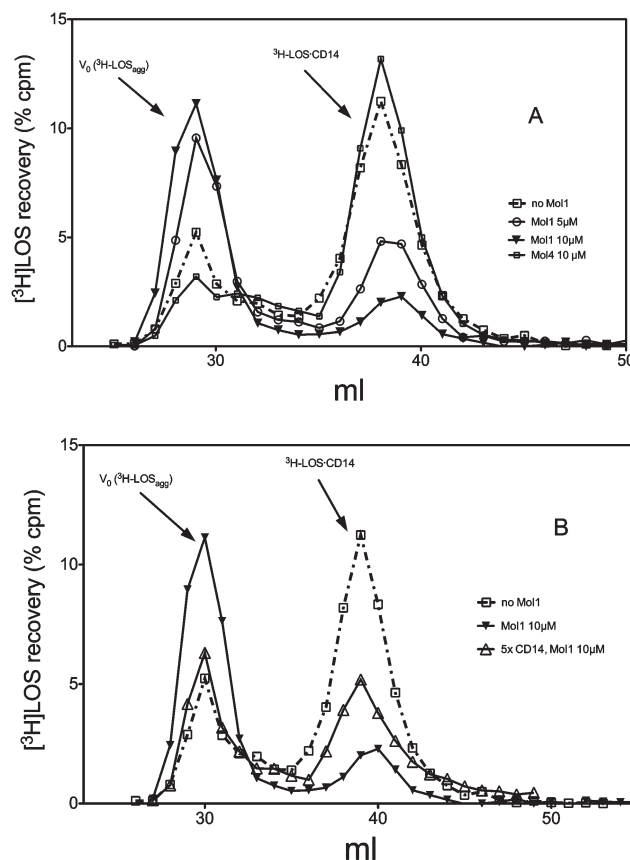


FIGURE 3: (A) Dose-dependent inhibition by molecule **1** but not by molecule **4** of LBP/sCD14-dependent extraction and transfer of $[^3\text{H}]\text{LOS}$ monomers from $[^3\text{H}]\text{LOS}$ aggregates to sCD14. sCD14 (0.8 nM) was preincubated with LBP (4 pM) and varying concentrations of the indicated synthetic molecules (0, 5, and 10 μM) for 30 min at 37 °C in PBS (pH 7.4) and 0.1% HSA. $[^3\text{H}]\text{LOS}_{\text{agg}}$ (0.8 nM) was then added to the reaction mixture followed by an incubation for 30 min at 37 °C, and reaction products were analyzed using Sephacryl S200 chromatography. (B) Inhibition by molecule **1** of transfer of $[^3\text{H}]\text{LOS}$ from LOS aggregates to sCD14 is attenuated by increasing sCD14 concentrations. Incubations and analysis were as described for panel A, except for the addition of a 5-fold higher sCD14 concentration (4 nM). The chromatographic profiles shown are representative of at least three experiments. Overall recoveries of $[^3\text{H}]\text{LOS}$ were $\geq 70\%$.

Figure 3A, molecule **1** caused a dose-dependent reduction in the extent of conversion of $[^3\text{H}]\text{LOS}_{\text{agg}}$ to $[^3\text{H}]\text{LOS}\cdot\text{sCD14}$, with a decreased extent of accumulation of $[^3\text{H}]\text{LOS}\cdot\text{sCD14}$ accompanied by an increased extent of recovery of $[^3\text{H}]\text{LOS}$ as high- M_r LOS aggregates (Figure 3A). In contrast, molecule **4**, a compound with a chemical structure similar to that of molecule **1** (Figure 1) but not active as an inhibitor of LPS-triggered TLR4 activation in vitro and in vivo (29, 30), had little or no effect on LBP/sCD14-dependent conversion of $[^3\text{H}]\text{LOS}_{\text{agg}}$ to $[^3\text{H}]\text{LOS}\cdot\text{sCD14}$ (Figure 3A). Thus, the ability of molecule **1**, but not molecule **4**, to inhibit LPS-triggered TLR4 signaling was paralleled by its ability to inhibit LBP/sCD14-dependent formation of the monomeric E·CD14 complex.

One possible mechanism by which molecule **1** inhibits formation of E·CD14 could be by competing with E (e.g., $[^3\text{H}]\text{LOS}$) for binding to CD14. In that case, increasing CD14 concentrations should reduce the inhibitory effect of molecule **1**. To test this hypothesis, we examined the effect of increasing the sCD14 concentration 5-fold on the ability of molecule **1** to inhibit conversion of $[^3\text{H}]\text{LOS}_{\text{agg}}$ to $[^3\text{H}]\text{LOS}\cdot\text{sCD14}$. A less inhibitory

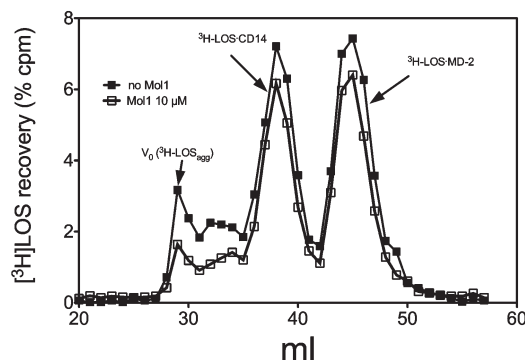


FIGURE 4: Molecule **1** does not inhibit transfer of [^3H]LOS monomers from CD14 to MD-2. sMD-2 (1.2 nM) containing medium was preincubated with sCD14 (0.8 nM) with or without molecule **1** for 30 min at 37 °C to give molecule **1** an opportunity to interact with MD-2. This preincubation mixture was then incubated for an additional 30 min at 37 °C with [^3H]LOS·sCD14 (0.8 nM) to allow for the transfer of [^3H]LOS to unoccupied MD-2. The reaction mixture products were analyzed by Sephacryl S200 chromatography. Chromatograms shown are representative of at least three independent experiments. Overall recoveries [^3H]LOS were > 70%.

effect of molecule **1** was observed under these conditions (Figure 3B), consistent with a direct competition between molecule **1** and LOS for CD14.

Molecule 1 Inhibits Binding of [^3H]LOS to CD14 but Not to MD-2. To test if molecule **1** can also inhibit transfer of [^3H]LOS monomers from CD14 to MD-2, we examined the effect of molecule **1** on this reaction. For this purpose, conditioned medium containing sMD-2 was preincubated with or without molecule **1** (10 μM) in the presence of LBP and sCD14 to allow formation of the CD14·molecule **1** complex possibly needed for the transfer of molecule **1** to MD-2. This preincubated mixture was then incubated with [^3H]LOS·sCD14 to measure the degree of transfer of LOS from sCD14 to MD-2 that had been preincubated with or without molecule **1** to assess the extent to which MD-2 is occupied by molecule **1**, inhibiting transfer of [^3H]LOS from CD14 to MD-2. Figure 4 shows that, in contrast to its effects on conversion of [^3H]LOS_{agg} to [^3H]LOS·sCD14, molecule **1** had little or no effect on the transfer of [^3H]LOS monomers from [^3H]LOS·sCD14 to MD-2, indicating that little or no MD-2 is occupied by molecule **1**.

The experiments described above demonstrate an ability of molecule **1** to inhibit extraction and transfer of [^3H]LOS monomers from [^3H]LOS aggregates to CD14 without affecting subsequent transfer of E monomers from [^3H]LOS·sCD14 to MD-2. This targeted inhibitory effect could be explained by a selective effect on extraction of E monomers from [^3H]LOS aggregates that is needed for generation of [^3H]LOS·sCD14 or a selective effect on net transfer of [^3H]LOS monomers to sCD14 versus sMD-2. To test the latter possibility more directly, we took advantage of the ability of monomeric E·sCD14 complexes to serve as a donor of E monomers to either CD14 or MD-2. For this purpose, we used [^3H]LOS·sCD14 (full-length sCD14, no His tag) as the [^3H]LOS donor and either His₆-tagged truncated sCD14 (tCD14; residues 1–156; 15) or His₆-tagged sMD-2 as the [^3H]LOS acceptor. Thus, transfer of [^3H]LOS from [^3H]LOS·sCD14 to tCD14-His₆ or to His₆-sMD-2 could be readily measured by an assay of the cocapture of [^3H]LOS with HISLINK resin as described in Experimental Procedures.

As expected, transfer of [^3H]LOS from [^3H]LOS·sCD14 to tCD14-His₆ and to His₆-sMD-2 was similar (Figure 5). Molecule

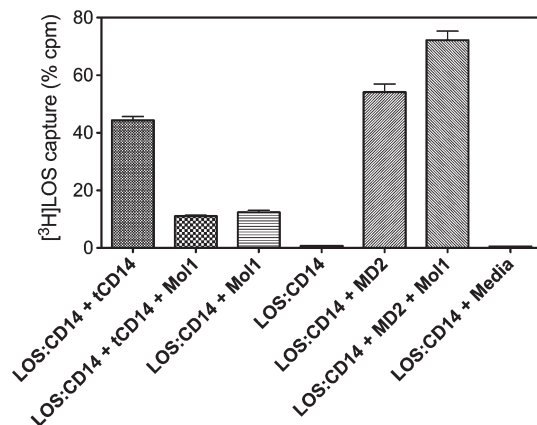


FIGURE 5: Molecule **1** selectively inhibits transfer of [^3H]LOS monomer to tCD14 but not to MD-2. His-tagged tCD14 or sMD2 (1.6 nM) was preincubated for 30 min at 37 °C with 10 μM molecule **1**, followed by addition of [^3H]LOS·sCD14 (0.8 nM) and further incubation for 30 min at 37 °C. Transfer of [^3H]LOS to His-tagged tCD14 and MD-2 was assayed by cocapture of [^3H]LOS to the HISLINK resin as described in Experimental Procedures. The degree of capture of [^3H]LOS·sCD14 (no His tag) before and after incubation in medium without His-tagged protein was < 4% and subtracted from each of the experimental samples shown. Data shown are representative of three experiments, each conducted in duplicate, and are expressed as means \pm the standard error of the mean.

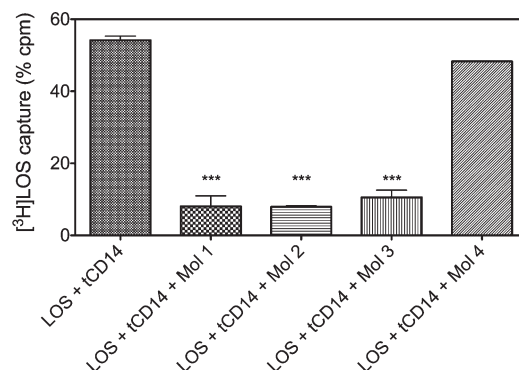


FIGURE 6: Molecules **1–3**, but not molecule **4**, promote displacement of [^3H]LOS from [^3H]LOS·tCD14-His₆. [^3H]LOS·tCD14-His₆ was formed by preincubation of tCD14-His₆ with [^3H]LOS_{agg} (0.8 nM) and LBP (4 pM) and then incubated for an additional 30 min at 37 °C with or without 10 μM molecules **1–4**, as indicated. The amount of remaining [^3H]LOS·tCD14-His₆ was assayed by cocapture of [^3H]LOS with HISLINK resin as described in Experimental Procedures. The level of nonspecific binding of [^3H]LOS_{agg} incubated in the absence of tCD14-His₆ was < 9% and subtracted from each of the experimental samples shown. Results shown represent the mean of three experiments each conducted in duplicate, and data are expressed as means \pm the standard error of the mean. Asterisks denote conditions under which incubation with the indicated synthetic molecule resulted in a statistically significant [$p < 0.01$ (ANOVA; Dunnett's test)] reduction in the extent of cocapture of [^3H]LOS (i.e., retention of [^3H]LOS·tCD14-His₆).

1 markedly inhibited transfer of [^3H]LOS from full-length sCD14 (no His tag) to tCD14-His₆ but did not inhibit transfer of [^3H]LOS to His₆-sMD-2 (Figure 5). Note that the presence of molecule **1** increased by $\sim 10\%$ the level of nonspecific capture of [^3H]LOS after incubation of LOS·sCD14 with or without MD-2 (Figure 5). This may be explained by the ability of molecule **1** to promote displacement of [^3H]LOS from LOS·sCD14 (see below), followed by nonspecific adsorption of some of the displaced monomeric [^3H]LOS to the HISLINK resin. These

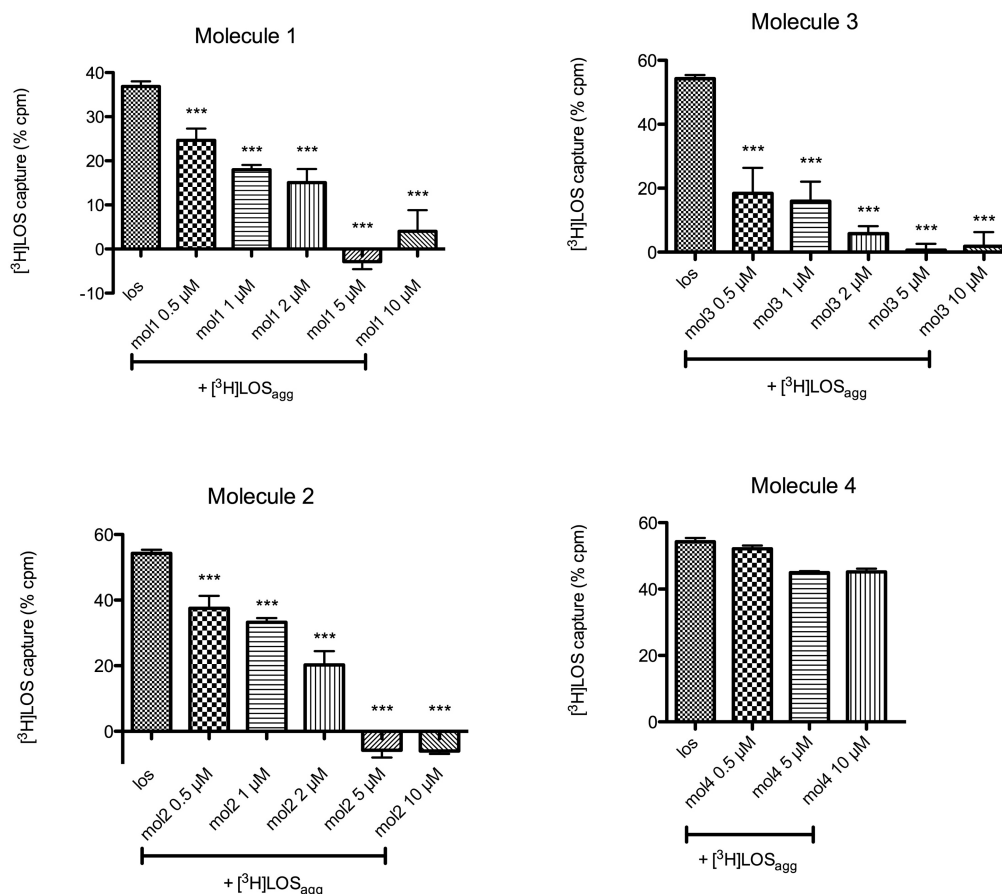


FIGURE 7: Dose-dependent inhibition of the net transfer of [³H]LOS monomers from [³H]LOS_{agg} to tCD14-His₆ by molecules 1–4 as assayed by cocapture of [³H]LOS with HISLINK resin. tCD14-His₆ (1.6 nM) was preincubated with LBP (4 pM) for 30 min at 37 °C with or without increasing concentrations of the indicated synthetic compounds. [³H]LOS_{agg} (0.8 nM) was then added, and samples were incubated for an additional 30 min at 37 °C. Results shown are the means ± the standard error of the mean of at least three independent experiments, each conducted in duplicate. Nonspecific binding, measured as [³H]LOS binding in absence of tCD14-His₆, was subtracted from each sample. Asterisks denote conditions under which preincubation of tCD14-His₆ with the added synthetic molecule resulted in a statistically significant [*p* < 0.01 (ANOVA; Dunnett's test)] reduction in the extent of cocapture of [³H]LOS (i.e., reduced net formation of [³H]LOS·tCD14-His₆).

results demonstrate that molecule 1 inhibits transfer (binding) of [³H]LOS monomer to CD14 but not to sMD-2.

Molecule 1 Promotes Displacement of [³H]LOS from [³H]LOS·CD14. Molecule 1 could inhibit net binding of [³H]LOS to CD14 by inhibiting transfer of [³H]LOS to CD14 and/or by promoting displacement of bound [³H]LOS from CD14. To test the latter possibility more directly, we incubated [³H]LOS_{agg} with tCD14 in the presence of LBP at 37 °C for 30 min in a PBS/0.1% albumin mixture, thus generating [³H]LOS·tCD14-His₆. This mixture containing the [³H]LOS·tCD14-His₆ complex was then incubated with or without 10 μM molecule 1 at 37 °C for 30 min. Loss of [³H]LOS from tCD14-His₆ was observed by both gel sieving chromatography (data not shown) and cocapture on the HISLINK resin (Figure 6). Both assays showed a marked diminution of [³H]LOS bound to tCD14-His₆ induced by incubation with molecule 1. Closely similar effects were seen with molecules 2 and 3, but not with molecule 4 (Figure 6), paralleling the ability of molecules 1–3, but not 4, at 1–10 μM, to inhibit LPS-triggered TLR4 signaling in vitro and in vivo (29, 30). We took advantage of this simpler and faster cocapture assay to quantify the inhibition of formation of the LOS·CD14 complex by our synthetic compounds (Figure 7). Molecules 1–3 showed a dose-dependent inhibition of the transfer of [³H]LOS monomers from [³H]LOS_{agg} to tCD14-His₆, with calculated IC₅₀ values of 0.8, 2, and 0.4 μM for molecules 1–3,

respectively. In contrast, molecule 4, even at 10 μM, produced little inhibition of the transfer of [³H]LOS monomers from [³H]LOS_{agg} to tCD14-His₆.

Saturation Transfer Difference (STD) NMR Shows Direct Binding between Molecule 1 and sCD14. The ability of molecule 1 to inhibit net transfer and/or binding of LOS to sCD14 could be most easily explained by an ability of molecule 1 (and related molecules 2 and 3) to bind to CD14 and thus weaken the interaction of LOS with CD14. To test more directly the possible interaction of molecule 1 with sCD14, we made use of STD NMR (33–35) in probing intermolecular interactions between molecule 1 and sCD14 and in identifying sites in molecule 1 that interact with sCD14. STD NMR experiments performed on the sample that contained both molecule 1 and sCD14 clearly showed saturation transfer from sCD14 to molecule 1, most strongly at 0.88 and 1.30 ppm and weakly at 3.13 ppm when the protein resonances were saturated by RF irradiation (Figure 8D). Identical STD NMR experiments performed on the control sample that contained only sCD14 showed no resonances at these chemical shift positions except for the broad peaks of background signals (Figure 8E). Excellent cancellation in the STD spectra of the strong solvent peaks present in the samples (Figure 8B–E) confirmed the good quality of the STD data. The STD peaks detected at 0.88 and 1.30 ppm are derived from the lipid chain CH₃ and CH₂ groups, respectively

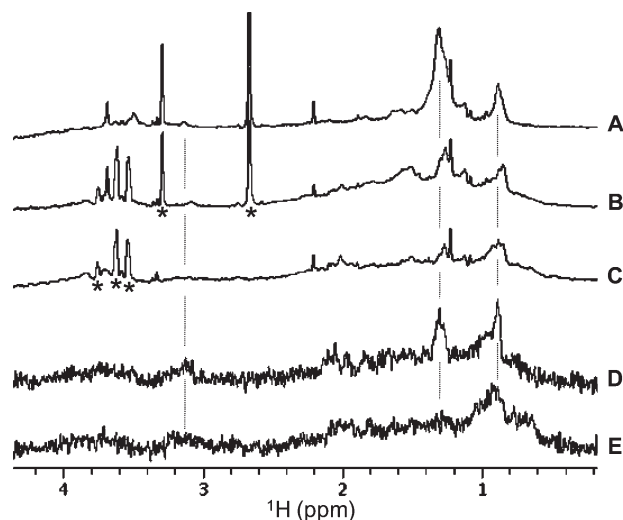


FIGURE 8: Saturation transfer difference (STD) NMR analysis of the interaction between molecule **1** and sCD14. (A–C) One-dimensional ^1H NMR spectra of 19 μM compound **1** (sample A), 19 μM compound **1** with 0.5 μM sCD14 (sample B), and 0.5 μM sCD14 (sample C), respectively. (D) STD NMR spectrum obtained for sample B. (E) STD NMR spectrum collected for control sample C. The stars denote ^1H peaks derived from solvents (DMSO at 2.66 ppm, methanol at 3.29 ppm) and/or buffer components (e.g., glycerol from sCD14 initial stock at 3.5–3.8 ppm).

(Figure 8A). The weak STD peak at 3.13 ppm is likely derived from the lipid chain OCH_2 group near the sugar moiety. These STD data strongly suggest that molecule **1** binds to CD14 and that the lipid chains have a major role in its interaction with CD14.

CONCLUSION

Previous experimental observations on molecule **1** and similar glycolipids or benzylammonium lipids suggested that the anti-endotoxic activity of these molecules is due to interference with the TLR4 pathway (29, 30). The lipid A-stimulated cytokine production by dendritic cells and macrophages is inhibited by compound **1**, and an antagonistic effect on TLR4-dependent but not on TLR2- or TLR9-dependent cell activation was observed in selectively transfected HEK293 cells (29). The main aim of this work was to improve our understanding of the mechanism of action of cationic glycolipid **1**, as a representative prototype of this class of compounds. For this purpose, we tested the effect of molecule **1** on the sequential extraction and transfer of $[\text{}^3\text{H}]\text{LOS}$ monomers from $[\text{}^3\text{H}]\text{LOS}_{\text{agg}}$ to LBP, CD14, and MD-2($\cdot\text{TLR4}_{\text{ECD}}$), reactions that are key to the efficient delivery of activating endotoxin to MD-2 $\cdot\text{TLR4}$ (12, 15). Our findings demonstrate that molecule **1**, and structurally related molecules **2** and **3**, affect this multistep pathway in a relatively selective manner, acting mainly to inhibit transfer to and/or stable occupation of CD14 by lipooligosaccharide ($[\text{}^3\text{H}]\text{LOS}$) monomers. Similar inhibitory effects of molecule **1** were seen when the transfer of $[\text{}^3\text{H}]\text{LOS}$ from either $[\text{}^3\text{H}]\text{LOS}$ aggregates or monomeric $[\text{}^3\text{H}]\text{LOS}\cdot\text{sCD14}$ was examined (Figures 3, 5, and 6), indicating that an additional effect of molecule **1** on interactions between LBP and E-rich interfaces was unlikely and not necessary for the inhibitory action of molecule **1**. Remarkably, shuttling of E monomers from CD14 to MD-2 is unaffected by molecule **1**. The apparently selective targeting of CD14 by these compounds bearing two lipid chains is

reminiscent of the previously described ability of CD14, but not MD-2, to interact stably with other compounds with lipid chains such as phospholipids (36) and lipopeptides (37, 38). The targeting of CD14 by molecules **1–3** is consistent with their ability to inhibit CD14-dependent TLR4 activation by endotoxin (lipid A/LPS) but not CD14-independent cell activation by TLR2 and TLR9 agonists (29). Moreover, as predicted from our biochemical studies, molecule **1** does not block TLR4 activation when endotoxin (LOS) is presented as a preformed $\text{LOS}\cdot\text{sCD14}$ complex that needs only to transfer LOS to MD-2 to induce TLR4 activation (data not shown).

Whether the selective inhibition of binding of LOS to CD14, but not to MD-2, reflects a higher affinity of these dialkylated compounds for CD14 versus MD-2 or rather a higher affinity and a more stable association of LOS with MD-2 than with CD14 cannot be judged. The inability of **1** to inhibit transfer of $[\text{}^3\text{H}]\text{LOS}$ to MD-2 from $[\text{}^3\text{H}]\text{LOS}\cdot\text{CD14}$ (Figures 4 and 5) underscores the efficiency of that transfer reaction and strongly suggests that **1** and related compounds must have time to occupy CD14 and thereby exclude E from CD14 to exert their maximum inhibitory effects. Although the STD NMR data did not provide the ligand binding site(s) on CD14 for the compound, these NMR data together with the fact that **1** inhibits the formation of the $\text{LOS}\cdot\text{sCD14}$ complex are most compatible with interaction of **1** with the hydrophobic cavity of CD14 believed to provide the binding site for endotoxins (lipid A) and a variety of other molecules, including triacylated lipids (37, 39).

Compounds **2** and **3**, but not **4**, were active in inhibiting the displacement of endotoxin from the complex with CD14, and compound **4** was also inactive in inhibiting CD14-mediated endotoxin presentation to MD-2 $\cdot\text{TLR4}$. The basic nitrogen present in compound **2** and the quaternary ammonium of **1** and **3** are replaced in **4** by the less basic methyl hydroxylamine. As a consequence, while sugar moieties of compounds **1–3** are cationic (compound **2** is protonated at neutral pH), compound **4** is mostly neutral under the conditions used for biological assays. The absence of a cationic group could affect dramatically the biological activity of **4**, influencing the aggregation state in solution as well the affinity for CD14. Work is in progress to determine the thermodynamic parameters of binding and affinity (including the stoichiometry of binding) between compounds **1–3** and CD14 and to further clarify the structural basis of the apparently lower reactivity of molecule **4** with CD14. The experiments reported here suggest that the affinity of compound **1** for CD14 is probably ~ 1000 -fold lower than that of LBP-treated E aggregates or monomeric E $\cdot\text{CD14}$ for CD14. The functional properties described suggest that molecules **1–3** could be considered lead compounds in the development of new antiendotoxic agents selectively targeting CD14. The broader role of CD14 (vs MD-2) in other biological recognition/response pathways may further expand the potential applications of these compounds.

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