Amino and Hydroxylamino Monosaccharides Inhibit lipid-A stimulated activation of Human Dendritic Cells and Macrophages

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Experimental Section

Biology: Dendritic cells, macrophages and culture medium. D1 cells were cultured in Iscove’s modified Dulbecco’s medium (IMDM, Sigma, St. Louis, MO) containing 10% heat-inactivated fetal bovine serum (Gibco-BRL, Gaithersburg, MD), 100 IU of penicillin, 100 µg/mL of streptomycin, 2mM L-glutamine (all from Sigma) and 50 µM β-mercaptoethanol (in complete IMDM) with 30% supernatant from R1 medium (supernatant from NIH3T3 fibroblasts transfected with GM-CSF). BMΦ and DC were derived from bone marrow cells collected from four different C57BL/6 mice (Harlan-Italy) and divided in two separate cultures. For DC preparation bone marrow cells were cultured in complete IMDM supplemented with 10% supernatant of GM-CSF–transduced B16 tumor cells.1 Fresh medium was added every 2 d. After 7–10 d of culture, cells were analyzed for CD11c expression and used in assays when 90% were CD11c positive. For BMΦ preparation bone marrow cells were cultured in complete IMDM supplemented with 10% supernatant of M-CSF–transduced NIH3T3 cells. Also in this case, fresh medium was added every 2 d. After 7–10 d of culture, cells were analyzed for Mac1 expression and used in assays when at least 90% were Mac1 positive.

Cell assays D1 cells, BMDC and MΦ were plated in 48 well plates at a concentration of 200,000 cells/well in 200 µL of medium. One or two hours after plating they were treated with different amounts of compounds 1 and 2 for 30 min and then stimulated with Lipid A from Escherichia coli (F583, Rd

mutant Sigma, 0.5µM) for 18 h. As control the cells were cultured in medium containing 0.25% (v/v) DMSO and 0.25% (v/v) EtOH, the same solvent mixture being used to dissolve monosaccharides.

**TNFα and IL-1β ELISA.** TNFα and IL-1β ELISA were performed using the DuoSet kit (R & D, Minneapolis, MN) and following the manufacturer recommendations.

**Assessment of apoptosis.** Cell apoptosis was assessed by flow cytometry. For murine cells, the exposure of phosphatidylserine residues on the cells' surface was measured using FITC-conjugated annexin V (BD PharMingen) and dead cells were identified with 1.25 µg/ml propidium iodide (Sigma-Aldrich).

**HEK-293-hTLR4A or –hTLR9A cell assay:** cells (InvivoGen, San Diego, CA, USA) were plated in 5 well plates at a concentration of 9.10^6 cells/well in 5mL of medium. Monosaccharide 2 was then added to the medium and after 2 h lipid A from *Escherichia coli* F583 (Rd mutant), Sigma-Aldrich (0.5 µM) or CpG (0.5 µM) was added to TLR4 or TLR9 transfected cells, respectively. After 2 h the medium was aspirated out of dish and cells were washed with 5mL of ice-cold PBS buffer containing a cocktail of phosphatase inhibitors supplied with the ELISA kit for the detection of NF-κB (Active Motif, Rixensart, Belgium). The buffer was then aspirated and 3 mL of the same buffer were added again. Cells were then transferred in a pre-chilled 15 mL conical tube, centrifuged (5 min, 1000 rpm) at 4 °C. Supernatant was discarded and cell pellet collected.

**Transcription factor NF-κB assay:** Transcription factor analysis was performed with an ELISA kit (Active Motif, Rixensart, Belgium) that allowed for the detection of NF-κB activation by a combination of NF-κB-specific oligonucleotide binding and subsequent detection of the p65 subunit of NF-κB with specific antibody. Cells were resuspended in 250 µL ice-cold hypotonic buffer (supplied with the nuclear extract kit, Active Motif, Rixensart, Belgium) supplemented with 25 µL of Nonidet P-40 and the mixture was centrifuged at 14000 x g for 2 min at 4°C. Pellets were suspended in 50 µL of hypertonic lysis buffer and incubated with shaking for 30 min at 4°C. Samples were then centrifuged at 14000 x g for 10 min at 4°C, and the supernatant containing nuclear extracts was stored at –80°C until use. Nuclear protein extract (10 µg) were added onto the oligonucleotide-coated ELISA plate and then incubated for 1 h at
room temperature. Primary antibody recognizing an epitope on p65, which is accessible only when NF-
κB is activated and bound to its target DNA, was added to wells and incubated for 1 h. This is followed
by the addition of an HPR-conjugated secondary antibody and, after 1 h, the HRP substrate was added.
The reaction was stopped after 5-10 min, and the absorbance was measured on a spectrophotometer
(Multiskan® EX, ThermolabSystem) at 450 nm. Jurkat cell nuclear extracts were used as an activated
NF-κB positive control. NF-κB wild-type and mutated consensus oligonucleotides were used in order to
monitor the specificity of the assay: a wild-type oligonucleotide should compete with NF-κB for binding,
whilst the mutated consensus oligonucleotide should have no effect on NF-κB binding.

**Chemistry, general procedures.** All solvents were dried over molecular sieves (4 Å, Fluka) for at least
24 h prior to use. When dry conditions were required, the reactions were performed under argon
atmosphere. Thin-layer chromatography (TLC) was performed on Silica Gel 60 F254 plates (Merck) with
UV detection, or using a developing solution of conc. H$_2$SO$_4$/EtOH/H$_2$O (5:45:45), followed by heating at
180°C. Flash column chromatography was performed on silica gel 230–400 mesh (Merck). Mixtures of
petroleum ether (boiling range 40–60°C) and ethyl acetate were used as eluent. $^1$H and $^{13}$C NMR spectra
were recorded on a Varian 400 MHz MERCURY instrument at 300 K. Chemical shifts are reported in
ppm downfield from TMS as internal standard; carbon signals of linear C$_{14}$ chains on C-2 and C-3 have
been omitted in the carbon spectra assignment.

Mass spectra were recorded on a Fourier Transform Ion Cyclotron Resonance (FT-ICR) instrument
(model APEXII, Bruker Daltonics), equipped with a 4.7 T Magnet (Magnex).

Optical rotations were measured at ambient temperature, using the sodium D line, on a P3002 electronic
polarimeter (A. Krüss, Germany).

**Methyl 6-deoxy-6-cyclopentylamino-4-O-(4’-methoxybenzyl)-2,3-di-O-tetradecyl-α-D-
glucopyranoside (8)** A stirred solution of aldehyde 7 (1.690 g, 2.4 mmol), cyclohexylamine (970 µL, 9.7
mmol), NaCNBH$_3$ (640 mg, 9.7 mmol) and AcOH (500 µL) in dry CH$_2$Cl$_2$/MeOH (2:1 v/v, 30 mL), was
warmed at 60 °C for 2 h under argon atmosphere. After this time the reaction was complete, according to
TLC analysis (7:3 toluene/EtOAc). The solvents were then evaporated *in vacuo*, the residue dissolved in
CH₂Cl₂ (50 mL) and washed with saturated aqueous bicarbonate and brine. The organic phase was dried over sodium sulphate, filtered and the solvent evaporated. The residue was purified by flash column chromatography on silica gel (9.5:0.5 EtOAc/MeOH) affording 7 (1.392 g, 75% yield) as a pale yellow powder. ¹H-NMR (CDCl₃) δ= 0.95 (m, 6H), 1.21 (m, 44H), 1.40-1.60 (m, 12 H), 2.62 (dd, 1H, J =12.1, 6.8 Hz), 2.84 (dd, 1H, J =12.1, 2.8 Hz), 3.00 (quintet, 1H, J =6.8 Hz), 3.26 (dd, 1H, J =9.6, 3.4 Hz), 3.31 (t, 1H, J = 9.3 Hz), 3.37 (s, 3H), 3.50-4.0 (m, 6H), 3.79 (s, 3H), 4.55 -4.80 (ABq, 2H), 4.71 (d, 1H, J=3.5 Hz), 6.80-7.20 (A₂X₂, 4H). ¹³C-NMR (CDCl₃) δ= 14.6, 23.1, 24.4, 26.4, 26.7, 29.8, 29.9-30.1 (signals of linear C₁₄ chain CH₂), 31.0, 32.4, 49.5, 55.59, 55.64, 59.9, 69.6, 72.0, 74.0, 74.8, 79.4, 81.1, 81.9, 98.1, 114.0, 129.9, 130.8, 159.4. HRMS (FT-ICR): calcd for C₄₈H₈₈NO₆: 774.6612 [M+1]; found: 774.6604.

Methyl 6-deoxy-6-cyclopentylamino-2,3-di-O-tetradecyl-α-D-glucopyranoside (1) Compound 8 (1.400 g, 1.9 mmol) was dissolved in TFA/CH₂Cl₂ (1:1 v/v, 20 mL) and stirred for 1 h. After this time, PMB hydrolysis was complete, as assessed by TLC analysis (EtOAc/MeOH, 9:1). Solvents were evaporated in vacuo, the residue dissolved in CH₂Cl₂ (50 mL) and washed with saturated aqueous bicarbonate and brine. The organic phase was dried over sodium sulphate, filtered and the solvent evaporated. The residue was purified by flash column chromatography on silica gel (9.5:0.5 EtOAc/MeOH) affording 1 (0.993 g, 80% yield) as a yellow powder. ¹H-NMR (CDCl₃) δ= 0.95 (m, 6H), 1.21 (m, 44H), 1.40-1.60 (m, 12 H), 2.59 (bs, 1H), 2.81 (dd, 1H, J =11.9, 7.6 Hz), 2.97 (dd, 1H, J =11.9, 4.9 Hz), 3.09 (quintet, 1H, J =6.6 Hz), 3.26 (dd, 1H, J =9.3, 3.5 Hz), 3.40 (s, 3H), 3.40-3.60 (m, 5H), 3.71 (m, 1H), 3.81 (m, 1H), 4.72 (d, 1H, J=3.6 Hz). ¹³C-NMR (CDCl₃) δ= 14.6, 23.1, 24.33, 24.37, 26.4, 26.5, 29.8, 29.9-30.1 (signals of linear C₁₄ chain CH₂), 30.8, 32.3, 33.1, 33.4, 51.7, 55.6, 60.3, 68.6, 71.9, 74.0, 75.6, 80.5, 81.2, 98.5. [α]₂⁰D: +18.4 (c 0.5, CHCl₃). (HRMS (FT-ICR): calcd for C₄₈H₈₈NO₆ [M+H]⁺: 654.6031, [M+Na]⁺: 676.5850; found: 654.6009; 676.5872.

Methyl 6-deoxy-6-N,N′,N″-dimethylcyclopentylamonium-2,3-di-O-tetradecyl-α-D-glucopyranoside (2) A solution of compound 1 (250 mg, 0.38 mmol), methyl iodide (50 µL, 0.76 mmol) and sodium carbonate (120 mg) in dry DMF (7 mL), was stirred under argon atmosphere at r.t. for 12 h. After this time reaction was complete (TLC, 7:3 EtOAc/MeOH). Solvent was evaporated in vacuo, the residue
dissolved in chloroform (15 mL) and washed with brine. The organic phase was dried over sodium sulphate, filtered and the solvent evaporated. Pure compound 2 (241 mg, 93% yield) was obtained as a white powder. \(^1\)H-NMR (CDCl\(_3\)) \(\delta = 0.80\) (bt, 6H, \(J = 6.8\) Hz), 1.21 (m, 44H), 1.51 (m, 4H), 1.66 (m, 1 H), 1.80 (m, 2H), 2.15 (m, 1H), 3.13 (dd, 1H, \(J = 9.8, 3.5\) Hz), 3.21 (s, 3H), 3.23 (s, 3H), 3.32 (bt, 1H, \(J = 9.2\) Hz), 3.42 (s, 3H), 3.40-3.55 (m, 4H), 3.68 (bt, 1H, \(J = 6.9\) Hz), 3.91 (bs, 1H), 4.05 (bt, 1H, \(J = 9.0\) Hz), 4.13 (bt, 1H, \(J = 8.5\) Hz), 4.19 (d, 1H, \(J = 14.1\) Hz), 4.66 (d, 1H, \(J = 3.5\) Hz). \(^13\)C-NMR (CDCl\(_3\)) \(\delta = 14.6, 23.1, 24.3, 24.33, 24.37, 26.4, 26.5, 29.8, 29.9-30.1\) (signals of linear C\(_{14}\) chain CH\(_2\)), 30.8, 32.3, 50.5, 57.8, 66.1, 67.3, 71.7, 72.3, 74.0, 76.7, 79.7, 80.2, 99.6. \(\alpha\)\(^{20}\)D: +21.6 (c 0.5, CHCl\(_3\)). HRMS (FT-ICR): calcd for C\(_{42}\)H\(_{85}\)NO\(_5\)\(^+\): 682.6344; found: 682.6354. Elemental analysis calcd, (%) for C\(_{42}\)H\(_{84}\)NO\(_5\)\(^+\): C 73.84, H 12.39, N 2.05; found: C 73.78, H 12.11, N 1.99.

**Methyl 6-deoxy-6-[N-(1’-tetrahydrofuranyl)-N’-methoxyamino]-2,3-di-O-tetradecyl-\(\alpha\)-D-glucopyranoside (3) To a solution of monosaccharide 10 (174 mg, 0.28 mmol) in dry CH\(_2\)Cl\(_2\), pyridinium \(p\)-toluenesulfonate (7 mg, 0.028 mmol) and dihydrofuran (64 \(\mu\)L, 0.42 mmol) were added under argon atmosphere and the mixture was stirred at r.t. for 3h. After this time, starting reagent was completely converted into the \(N,O\) di-THF adduct as assessed by TLC analysis (toluene/EtOAc, 7:3). Solvent was evaporated and the residue dissolved in AcOH/THF/H\(_2\)O 4:4:1 (9 mL). The conversion of the \(bis\)-THF adduct into 3 was followed by TLC and was complete after 6h. Solvents were then evaporated in vacuo, and the residue was purified by flash column chromatography on silica gel (AcOEt/petroleum ether, gradient of polarity starting from 2.5: 7.5) affording 3 as a white powder (129 mg, 68% yield). \(^1\)H-NMR (CDCl\(_3\)): \(\delta (ppm) = 0.85\) (t, 6H, \(J = 5.8\) Hz), 1.22 (m, 44H), 1.50-1.60 (m, 4H), 1.72 (m, 2H), 1.91 (m, 2H), 2.78 (bt, 1H, H-6), 3.11 (bt, 1H, H-4), 3.23 (dd, 1H, \(J = 9.4, 3.6\) Hz, H-6), 3.40 (s, 3H, NOCH\(_3\)), 3.50-3.70 (m, 3 H), 3.90 (bm, 2H, H-4’), 4.20 (bm, 1H, H-1’), 4.73 (d, 1H, \(J = 3.6\) Hz, H-1). \(^13\)C-NMR (400 MHz, CDCl3): \(\delta(ppm) = 97.5, 92.03, 80.65, 79.20, 76.42, 72.08, 70.84, 61.27, 59.92, 58.71, 53.89, 30.60, 29.0-28.0\) (bulk of CH\(_2\) signals), 21.39, 12.84. \(\alpha\)\(^{20}\)D = +42.45° (c = 0.5, MeOH). HRMS (FT-ICR): calcd for C\(_{40}\)H\(_{79}\)NO\(_7\)\(^+\): 685.5857; found: 708.5710 [M+Na]\(^+\). Elemental analysis calcd, (%) for C\(_{40}\)H\(_{79}\)NO\(_7\) : C 70.03, H 11.61, N 2.04; found: C 70.07, H 11.57, N 2.01.