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New design of cationic alkyl glycoglycerolipids toxic to tumor cells

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General methods

All solvents for column chromatography were distilled before using. Molecular sieves were activated at 180°C for 2 h *in vacuo*. Thin layer chromatography was performed using pre-coated aluminum plates (Kieselgel 60 F_{254}, Merck), which were visualized with the phosphomolybdic acid–ceric sulfate reagent. Flash column chromatography was performed on Kieselgel 60 (40–63 μm, Merck). ^{1}H and ^{13}C NMR spectra were recorded at 24°C on a Bruker DPX-300 spectrometer in $CDCl_3$ as a solvent. The signals of $CDCl_3$ (^{1}H δ 7.26 and ^{13}C δ 77.16 ppm) were used as internal reference. *J* values are given in Hz. Mass spectra were recorded on a Bruker Ultraflex time-of-flight mass spectrometer (MALDI-TOF MS) using 2,5-dihydroxybenzoic acid as a matrix.

Synthesis of compounds 5a,b (general procedure): Mesyl chloride (14.4 μl, 0.187 mmol) in anhydrous pyridine (4 ml) was cooled to -15˚C and added to a cooled to -15˚C solution of 1-*O*-octadecyl-2-*O*-ethyl-3-*O*-β-D-galactopyranosylglycerol (0.100 g, 0.187 mmol) or 1-*O*-octadecyl-2-*O*-ethyl-3-*O*-α-D-mannopyranosylglycerol (0.100 g, 0.187 mmol) in anhydrous pyridine (4 ml). The reaction mixture was held at -15 ˚C for 2 d. Then acetic anhydride (70.6 μl, 1.122 mmol) was added and the mixture was stirred at 20 ˚C for 24 h. Then the reaction mixture was diluted by chloroform (20 ml), washed with 3% aq. solution of HCl (3×40 ml) and water (40 ml). The organic layer was dried over Na_2SO_4, filtered, and the solvent was evaporated. The product was isolated by column chromatography (toluene–ethyl acetate, 6:1 → 4:1).

2-*O*-ethyl-1-*O*-octadecyl-3-*O*-(2,3,4-tri-*O*-acetyl-6-*O*-methanesulfonyl-β-D-galactopyranosyl)glycerol 5a: Yield 46%. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 0.87 (t, 3H, $(CH_2)_{15}$*Me*, *J* 6.7 Hz), 1.17 and 1.18 (2t for two diastereoisomers, 3H, OCH_2*Me*, *J* 7.0 Hz), 1.25 (br. s, 30H, $(CH_2)_{15}$Me), 1.46–1.60 (m, 2H, OCH_2CH_2), 1.98 (s, 3H), 2.05 (s, 3H) and 2.16 (s, 3H, 3OCOMe), 3.02 and 3.03 (2s for two diastereoisomers, 3H, SO_2Me), 3.33–3.49 (m, 4H, OCH_2CH_2, OCH_2), 3.51–3.69 (m, 4H, CH(O), OCH_a, OCH_2Me), 3.81–4.03 (m, 2H, OCH_b, H-5 Gal), 4.12–4.35 (m, 2H, H-6 Gal), 4.54 and 4.58 (2d for two diastereoisomers, 1H, H-1 Gal, *J* 7.9 Hz), 5.00 and 5.03 (2dd for two diastereoisomers, 1H, H-3 Gal, *J* 3.4, 1.6 Hz), 5.12–5.30 (m, 1H, H-2 Gal), 5.39–5.44 (m, 1H, H-4 Gal). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 14.2, 15.7, 15.8, 20.7, 20.8, 22.8, 29.5, 29.6, 29.8, 32.0, 37.8, 65.8, 66.2, 67.0, 68.8, 69.6, 70.2, 70.4, 70.7, 70.8, 70.9, 71.0, 71.9, 77.3, 77.8, 101.7, 101.9, 169.4, 169.5, 170.2, 170.4. MS, *m/z*: 761.201 $[M+Na]^+$. Calc. for $C_{36}H_{66}O_{13}SNa$: 761.4122 $[M+Na]^+$.

2-*O*-ethyl-1-*O*-octadecyl-3-*O*-(2,3,4-tri-*O*-acetyl-6-*O*-methanesulfonyl-α-D-mannopyranosyl)glycerol 5b: Yield 93 %. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 0.87 (t, 3H, $(CH_2)_{15}$*Me*, *J* 6.7 Hz), 1.18 and 1.19 (2t for two diastereoisomers, 3H, OCH_2*Me*, *J* 7.0 Hz), 1.24 (br. s, 30H, $(CH_2)_{15}$Me), 1.48–1.60 (m, 2H, OCH_2CH_2), 1.98 (s, 3H), 2.05 (s, 3H) and 2.14 (s,

3H, 3 OCOMe), 3.06 (s, 3H, SO_2Me), 3.37–3.85 (m, 9H, CH_2OCH_2, $CHOCH_2$, $OCH\mathit{H}_a$, H-5 Man), 4.06–4.21 (m, 1H, H_a-6 Man), 4.24–4.35 (m, 2H, H_b-6 Man, $OCH\mathit{H}_b$), 4.84 and 4.87 (2d for two diastereoisomers, 1H, H-1 Man, *J* 1.7 Hz), 5.20–5.39 (m, 3H, H-2, H-3, H-4 Man). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 14.2, 15.7, 20.8, 21.0, 22.8, 26.2, 29.5, 29.6, 29.7, 29.8, 32.0, 37.8, 37.9, 66.1, 67.6, 67.7, 68.1, 68.4, 68.5, 69.0, 69.1, 69.5, 70.0, 71.9, 72.0, 77.4, 97.5, 98.0, 169.9, 170.0, 170.1. MS, *m/z*: 761.201 $[M+Na]^+$. Calc. for $C_{36}H_{66}O_{13}SNa$: 761.4122 $[M+Na]^+$.

Synthesis of compounds 6a,b (general procedure): A solution of compound **5a** or **5b** (0.0523 g, 0.070 mmol) in anhydrous pyridine (10 ml) was refluxed at 115 °C for 5 h and evaporated *in vacuo*. The product was isolated by column chromatography (chloroform–methanol–1% aq. CH_3COOH, 40:10:1).

2-*O*-ethyl-1-*O*-octadecyl-3-*O*-(2,3,4-tri-*O*-acetyl-6-deoxy-6-pyridinio-β-D-galactopyranosyl)glycerol acetate 6a: Yield 45%. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 0.87 (t, 3H, $(CH_2)_{15}\mathit{Me}$, *J* 6.7 Hz), 1.17 and 1.18 (2t for two diastereoisomers, 3H, $OCH_2\mathit{Me}$, *J* 7.0 Hz), 1.26 (br. s, 30H, $(C\mathit{H}_2)_{15}Me$), 1.48–1.63 (m, 2H, $OCH_2C\mathit{H}_2$), 1.99 (s, 6H), 2.06 (s, 3H) and 2.23 (s, 3H, 4OCOMe), 3.25–3.69 (m, 9H, CH_2OCH_2, $CHOCH_2$, OCH_2), 4.36–4.71 (m, 3H, H-1, H_a-6, H-5 Gal), 5.02–5.16 (m, 3H, H_b-6, H-3, H-2 Gal), 5.60–5.63 (m, 1H, H-4 Gal), 8.05–8.17 (m, 2H), 8.50–8.62 (m, 1H) and 9.03–9.15 (m, 2H, Py). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 13.8, 15.2, 20.2, 20.4, 22.5, 25.8, 29.2, 29.3, 29.4, 29.5, 31.7, 47.8, 48.1, 48.3, 48.6, 48.9, 49.2, 49.5, 61.2, 65.7, 65.9, 67.8, 68.5, 68.6, 69.4, 69.7, 69.8, 69.9, 70.4, 70.5, 71.5, 71.7, 76.9, 100.9, 101.1, 127.8, 127.9, 145.7, 145.8, 145.9, 169.7, 169.8, 170.0, 170.3. MS, *m/z*: 722.419 $[M\text{-}AcO^-]^+$. Calc. for $C_{40}H_{68}NO_{10}$: 722.4843 $[M\text{-}AcO^-]^+$.

2-*O*-ethyl-1-*O*-octadecyl-3-*O*-(2,3,4-tri-*O*-acetyl-6-deoxy-6-pyridinio-α-D-mannopyranosyl)glycerol acetate 6b: Yield 96 %. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 0.87 (t, 3H, $(CH_2)_{15}\mathit{Me}$, *J* 6.7 Hz), 1.11–1.22 (m, 3H, $OCH_2\mathit{Me}$), 1.25 (br. s, 30H, $(C\mathit{H}_2)_{15}Me$), 1.44–1.61 (m, 2H, $OCH_2C\mathit{H}_2$), 2.00 (s, 3H), 2.08 (s, 3H) and 2.20 (s, 6H, 4OCOMe), 3.25–3.71 (m, 11H, CH_2OCH_2, $CHOCH_2$, OCH_2, H-5, H_a-6 Man), 4.26–4.90 (m, 2H, H_b-6, H-1 Man), 4.94–5.39 (m, 3H, H-2, H-3, H-4 Man), 8.02–8.16 (m, 2H), 8.49–8.62 (m, 1H), 8.94–9.14 (m, 2H, Py). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 13.8, 15.2, 15.3, 20.2, 20.3, 20.4, 20.5, 22.5, 25.9, 29.2, 29.3, 29.4, 29.5, 30.6, 31.8, 65.7, 65.8, 65.9, 66.3, 66.5, 67.8, 68.3, 68.5, 68.6, 68.7, 68.9, 69.4, 71.5, 71.7, 77.0, 97.0, 97.8, 127.7, 127.9, 145.9, 169.9, 170.3, 170.6, 170.7. MS, *m/z*: 722.513 $[M\text{-}AcO^-]^+$. Calc. for $C_{40}H_{68}NO_{10}$: 722.4843 $[M\text{-}AcO^-]^+$.

Synthesis of compounds 7a,b (general procedure): A solution of compound **5a** or **5b** (0.046 g, 0.06 mmol) in anhydrous methyl ethyl ketone (4 ml) was refluxed with anhydrous *N*-methylimidazole (0.01 ml) at 80 °C for 2 d, then evaporated *in vacuo*. The product was isolated by column chromatography (chloroform–methanol–1% aq. CH_3COOH, 40:10:1).

2-*O*-ethyl-1-*O*-octadecyl-3-*O*-(2,3,4-tri-*O*-acetyl-6-deoxy-6-*N*-methylimidazolio-β-D-galactopyranosyl)glycerol acetate 7a: Yield 34 %. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 0.87 (t, 3H, $(CH_2)_{15}\mathit{Me}$, *J* 6.7 Hz), 1.17 and 1.18 (2t for two diastereoisomers, 3H, $OCH_2\mathit{Me}$, *J* 7.0), 1.25 (br. s, 30H, $(C\mathit{H}_2)_{15}Me$), 1.48–1.61 (m, 2H, $OCH_2C\mathit{H}_2$), 1.98 (s, 6H), 2.05 (s, 3H) and 2.20 (s, 3H, 4 OCOMe), 3.21–3.85 (m, 11H, CH_2OCH_2, $CHOCH_2$, OCH_2, H_a-6, H-5 Gal), 3.97 (s, 3H, NMe), 4.44–4.63 (m, 2H, H_b-6, H-1 Gal), 5.04–5.29 (m, 2H, H-2, H-3 Gal), 5.44–5.53 (m, 1H, H-4 Gal), 7.37–7.41 (m, 1H) and 7.46–7.53 (m, 1H, CH=CH). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 13.7, 15.2, 20.1, 20.2, 20.4, 22.4, 25.8, 29.1, 29.3, 29.4, 29.5, 31.7, 36.0, 38.8, 49.6, 65.6, 65.8, 67.9,

68.6, 69.5, 69.8, 70.5, 71.4, 71.6, 71.7, 77.0, 77.2, 100.9, 101.1, 123.0, 123.3, 123.4, 169.7, 169.8, 170.0, 170.3. MS, *m/z*: 726.473 $[M\text{-}AcO^-+H]^+$. Calc. for $C_{39}H_{70}N_2O_{10}$: 726.5025 $[M\text{-}AcO^-+H]^+$.

2-*O*-ethyl-1-*O*-octadecyl-3-*O*-(2,3,4-tri-*O*-acetyl-6-deoxy-6-*N*-methylimidazolio-α-D-mannopyranosyl)glycerol acetate 7b: Yield 87 %. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 0.87 (t, 3H, $(CH_2)_{15}$*Me*, *J* 6.7 Hz), 1.13–1.22 (m, 3H, OCH_2*Me*), 1.24 (br. s, 30H, $(CH_2)_{15}$Me), 1.45–1.61 (m, 2H, OCH_2CH_2), 1.97 (s, 6H), 2.07 (s, 3H) and 2.15 (s, 3H, 4OCOMe), 3.38–3.76 (m, 9H, CH_2OCH_2, $CHOCH_2$, OCH_2), 3.97 (s, 3H, NMe), 4.13–4.33 (m, 1H, H-5 Man), 4.40–4.54 (m, 2H, H-6 Man), 4.81–5.00 (m, 2H, H-1, H-2 Man), 5.20–5.27 (m, 1H, H-4 Man), 5.28–5.39 (m, 1H, H-3 Man), 7.41–7.52 (m, 1H) and 7.52–7.62 (m, 1H, CH=CH). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 13.6, 15.1, 20.1, 20.2, 22.4, 25.7, 29.1, 29.2, 29.3, 29.4, 31.6, 35.8, 38.7, 49.1, 65.7, 65.8, 65.9, 66.0, 68.0, 68.2, 68.5, 68.9, 69.4, 71.6, 76.8, 77.1, 97.0, 97.7, 122.9, 123.2, 169.7, 169.8, 169.9, 170.5. MS, *m/z*: 724.969 $[M\text{-}AcO^-]^+$. Calc. for $C_{39}H_{69}N_2O_{10}$: 725.4952 $[M\text{-}AcO^-]^+$.

Synthesis of compounds 8a,b and 9a,b (general procedure): A 0.1 M solution of MeONa in methanol (8 ml) was added to a solution of compound **6a,b** or **7a,b** (0.0588 mmol) in dichloromethane (10 ml). After 1 h, the reaction mixture was neutralized with Dowex 50W×8 ion-exchange resin (H^+) and filtered, the solvent was evaporated *in vacuo*. The product was isolated by column chromatography with CH_3Cl–MeOH–1% aq. CH_3COOH solvent system (20:10:1).

2-*O*-ethyl-1-*O*-octadecyl-3-*O*-(6-deoxy-6-pyridinio-β-D-galactopyranosyl)glycerol acetate 8a: Yield 85 %. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 0.87 (t, 3H, $(CH_2)_{15}$*Me*, *J* 6.7 Hz), 1.16 and 1.18 (2t for two diastereoisomers, 3H, OCH_2*Me*, *J* 7.0 Hz), 1.24 (br. s, 30H, $(CH_2)_{15}$Me), 1.46–1.61 (m, 2H, OCH_2CH_2), 2.02 (s, 3H, OCOMe), 3.29–3.69 (m, 12H, CH_2OCH_2, $CHOCH_2$, OCH_2, H-2, H-3, H-5 Gal), 4.04–4.23 (m, 2H, H-1, H-4 Gal), 4.77–4.92 (m, 1H, H_a-6 Gal), 5.09–5.23 (m, 1H, H_b-6 Gal), 8.00–8.11 (m, 2H), 8.43–8.55 (m, 1H) and 9.07–9.19 (m, 2H, Py). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 13.9, 15.2, 22.5, 25.9, 29.2, 29.4, 29.5, 29.6, 31.8, 62.3, 65.6, 65.8, 68.5, 68.7, 69.1, 69.9, 70.2, 70.4, 70.5, 71.7, 72.6, 73.1, 73.2, 77.2, 103.5, 103.6, 127.6, 145.4, 145.8, 169.3, 170.5. MS, *m/z*: 596.341 $[M\text{-}AcO^-]^+$. Calc. for $C_{34}H_{62}NO_7$: 596.4526 $[M\text{-}AcO^-]^+$.

2-*O*-ethyl-1-*O*-octadecyl-3-*O*-(6-deoxy-6-pyridinio-α-D-mannopyranosyl)glycerol acetate 8b: Yield 54 %. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 0.87 (t, 3H, $(CH_2)_{15}$*Me*, *J* 6.7 Hz), 1.13 and 1.14 (2t for two diastereoisomers, 3H, OCH_2*Me*, *J* 7.0 Hz), 1.25 (br. s, 30H, $(CH_2)_{15}$Me), 1.41–1.62 (m, 2H, OCH_2CH_2), 1.97 (s, 3H, MeCOO), 2.99–4.09 (m, 13H, CH_2OCH_2, $CHOCH_2$, OCH_2, H-2, H-3, H-4, H-5 Man), 4.67–4.82 (m, 1H, H-1 Man), 4.93–5.23 (m, 2H, H-6), 8.00–8.19 (m, 2H), 8.37–8.55 (m, 1H), 9.05–9.22 (m, 2H, Py). MS, *m/z*: 596.387 $[M\text{-}AcO^-]^+$. Calc. for $C_{34}H_{62}NO_7$: 596.4526 $[M\text{-}AcO^-]^+$.

2-*O*-ethyl-1-*O*-octadecyl-3-*O*-(6-deoxy-6-*N*-methylimidazolio-β-D-galactopyranosyl)glycerol acetate 9a: Yield 57 %. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 0.87 (t, 3H, $(CH_2)_{15}$*Me*, *J* 6.7 Hz), 1.18 and 1.19 (2t for two diastereoisomers, 3H, OCH_2*Me*, *J* 7.0 Hz), 1.25 (br. s, 30H, $(CH_2)_{15}$Me), 1.49–1.52 (m, 2H, OCH_2CH_2), 1.96 (s, 3H, OCOMe), 3.24–3.92 (m, 14H, CH_2OCH_2, $CHOCH_2$, OCH_2, H-1, H-2, H-3, H-4, H-5 Gal), 3.91 (s, 3H, NMe), 4.35–4.49 (m, 1H, H_a-6 Gal), 4.72–4.85 (dd, 1H, H_b-6 Gal, *J* 12.7, 1.6 Hz), 7.48–7.50 (m, 1H) and 7.53–7.57 (m, 1H, CH=CH). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 13.6, 15.0, 22.3, 25.7, 29.0, 29.2, 29.3, 29.4, 31.6, 35.7, 38.5, 50.0, 65.6, 65.8, 66.5, 66.6, 67.4, 67.5, 69.8, 69.9, 70.0, 70.6, 70.7, 71.5, 71.6, 77.0, 100.2, 100.6, 122.8, 123.6. MS, *m/z*: 599.038 $[M\text{-}AcO^-]^+$. Calc. for $C_{33}H_{63}N_2O_7$: 599.4635 $[M\text{-}AcO^-]^+$.

2-*O*-ethyl-1-*O*-octadecyl-3-*O*-(6-deoxy-6-*N*-methylimidazolio-α-D-mannopyranosyl)glycerol acetate 9b: Yield 61 %. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 0.87 (t, 3H, $(CH_2)_{15}$*Me*, *J* 6.7 Hz), 1.17 (t, 3H, OCH_2*Me*, *J* 7.0 Hz), 1.24 (br. s, 30H, $(CH_2)_{15}$Me), 1.46–1.62 (m, 2H, OCH_2CH_2), 1.92 (s, 3H, OCOMe), 3.20–3.88 (m, 14H, CH_2OCH_2, $CHOCH_2$, OCH_2, H-2, H-3, H-4, H-5 Gal), 3.93 (s, 3H, NMe), 4.32–4.45 (m, 2H, H-6 Gal), 4.76 and 4.81 (2d for two diastereoisomers, 1H, H-1 Man, *J* 1.4 Hz), 7.33–7.42 (m, 1H) and 7.51–7.59 (m, 1 H, CH=CH). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 13.6, 15.0, 22.4, 25.8, 29.1, 29.2, 29.3, 29.4, 31.6, 35.7, 50.0, 50.1, 65.7, 65.8, 66.4, 66.5, 67.4, 67.6, 69.8, 69.9, 70.0, 70.1, 70.5, 70.6, 71.6, 77.0, 100.3, 100.6, 122.7, 122.8, 123.7. MS, *m/z*: 599.057 $[M\text{-}AcO^-]^+$. Calc. for $C_{33}H_{63}N_2O_7$: 599.4635 $[M\text{-}AcO^-]^+$.

Cell culture and cytotoxicity assay

K562 and HL60 human leukemia, HCT116 colon carcinoma and B16 murine melanoma cells as well as human postnatal fibroblast cells were cultured in Dulbecco modified Eagle's medium supplemented with 5% fetal calf serum (HyClone, Logan, UT), 2 mM L-glutamine, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin at 37 ºC and 5% CO_2 in humidified atmosphere. The cells in logarithmic phase of growth were used for the experiments.

The synthesized compounds and edelfosine were dissolved in dimethyl sulfoxide as 10 mM stock solutions, followed by serial dilutions in culture medium immediately before experiments. Assays were performed in 96-well microtiter plates. To each well containing 5–7.5×10^4 tumor cells, the required amount of the test compound was added. The cells were subjected to incubation at 37 ºC and 5% CO_2 for 72 h, followed by the addition of aqueous solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (final concentration 0.25 mg ml^{-1}) and incubation for another 1–2 h. After the incubation was complete, the medium was removed, the cells were resuspended in dimethyl sulfoxide (100 μl) to measure the optical density of the solutions on a Multiscan FC tablet spectrophotometer (ThermoScientific, USA) at 570 nm. The percentage of cells survived upon treatment with certain concentration of test compound was calculated as the ratio of optical density in the wells (an average of three measurements) after incubation with this concentration of the test compound to average optical density in control wells taken as 100%

Hemolytic activity assay

Erythrocytes were obtained from peripheral blood of healthy persons. The blood was collected in the test-tubes containing 1% sodium citrate, stirred, and incubated at 4 ºC for 1 h. The suspension of erythrocytes was washed with buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4), the cells were suspended in 50 μl of the same buffer. Test compounds were added to the final concentrations given in Table 2. The volume of the added solutions of test compounds did not exceed 10% of a total volume of the mixture. In the control test-tube, distilled water (50 μl) was added to the suspension of erythrocytes. The samples were incubated at 37 ºC for 1 h with gentle stirring, then centrifuged at 2000 rpm. Optical density of the supernatant was measured on a LKB spectrophotometer (Sweden) at 545 nm. The obtained values of optical density were compared with the data for hemolysis induced by water (the absorption was taken as 100%). The experiments were performed four times with essentially the same results.