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Changping Zheng, Huimin Guan, Yan Liu, Zhihao Li, Teodora Bavaro, et al.. Chemoenzymatically synthesized ganglioside GM3 analogues with inhibitory effects on tumor cell growth and migration. *European Journal of Medicinal Chemistry*, 2019, 165, pp.107-114. 10.1016/j.ejmech.2019.01.016 . hal-02169381

HAL Id: hal-02169381

<https://hal.sorbonne-universite.fr/hal-02169381>

Submitted on 1 Jul 2019

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Chemoenzymatically synthesized ganglioside GM3 analogues with inhibitory effects on tumor cell growth and migration

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Abstract

Ganglioside GM3, belonging to glycosphingolipid family, has been known as tumor-associated carbohydrate antigen on several types of tumor. Many studies have revealed that GM3 plays a role in cell proliferation, adhesion and differentiation, which is crucial in the process of cancer development. In the present study, we firstly synthesized novel mannose containing GM3 analogues by enzymatic hydrolysis and chemical procedures. Then the antiproliferative activity of the novel analogues along with galactose containing analogues we prepared previously was investigated and the data demonstrated that these analogues exhibited antiproliferative effect on K562 and HCT116 cells. Finally, the influence of these analogues on tumor cell migration was studied on B16, B16-F10 and HCCLM3 cells by wound healing test, because the migration of tumor cells represents one of the relevant factors in assessing the malignancy of cancer. This study could lay the foundation for optimizing leading compounds and provide valuable information for finding new antitumor drugs for cancer therapy.

Keywords: GM3 analogues; chemoenzymatic synthesis; cancer; antitumor; tumor cell migration.

1. Introduction

Glycosphingolipids (GSLs) usually exist on the outer surface of cell membranes, and especially, they are the major glycolipids of humans. A variety of studies have demonstrated their biological roles in membrane structure, host–pathogen interactions, cell-cell recognition, and modulation of membrane protein function [1,2]. Ganglioside GM3, the first and simplest member in the metabolic series of a GSLs family, contains a single terminal sialic acid, lactose and ceramide (**Figure 1**). It has been reported that GM3 is implicated in the progression of human cancer development [3-5].

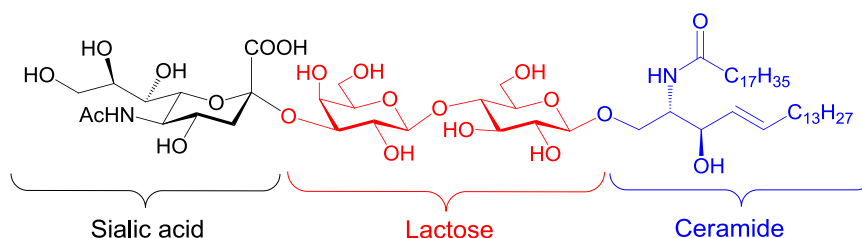


Figure 1. Chemical structure of ganglioside GM3.

Ganglioside GM3 is over expressed on some cancers, and is well known as the tumor-associated carbohydrate antigen on several types of tumor, such as melanoma and bladder cancers [6,7]. The obvious influence of GM3 on the proliferation, invasion and metastasis of tumor cells has been observed. GM3 can not only modulate the receptors including epidermal growth factor receptor (EGFR) [8], vascular endothelial growth factor receptor-2 (VEGFR-2) [9], but also glycolipid-enriched membrane microdomain signaling pathway [10]. Besides, some studies have revealed that GM3 has a clear effect on tumor cell movement, such as motility and migration [11,12].

It has been confirmed that cell movement in tumor growth is crucial because it is not only believed to influence the tumor shape, but also together with cell to cell adhesive properties, and it represents one of the relevant factors in assessing the malignancy of cancer. Previous studies suggested that a potential therapy can be represented by pharmacological agents that block tumor motility [13,14].

So far, some GM3 analogues have been synthesized [15], and in order to find

better antitumor agents, investigation on GM3 analogues with antitumor activities appears to be a logical matter of research. In the present study, the novel mannose containing analogues C1 and C2 (**Figure 2**) were prepared by chemoenzymatic synthesis. Then along with galactose containing analogues C3 and C4 (**Figure 2**) we prepared previously [16], their antitumor activities were evaluated by cytotoxicity assay. Furthermore, the influence of these four analogues on tumor cell migration was studied.

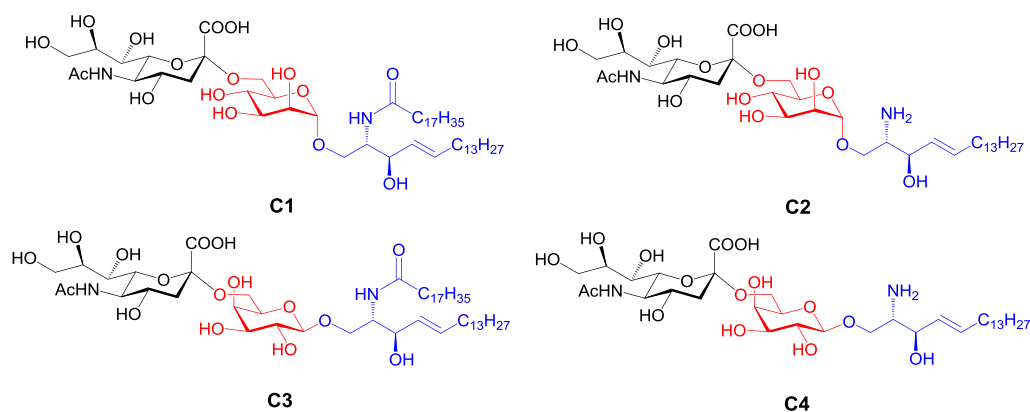


Figure 2. Structure of four synthesized GM3 analogues, including compound C1, C2, C3 and C4, respectively.

2. Results and discussion

2.1. Synthesis of GM3 analogues

For synthesizing GM3 analogues C1 and C2, according to the retrosynthetic analysis, three building blocks are needed (**Figure 3**). Firstly, the sialic acid was activated as the sialyl xanthate form based on the previous report [17]. Then the mannose bearing a free 6-OH residue M1 was obtained by enzymatic hydrolysis according to the reported method [18,19]. In this study, we introduced enzymatic hydrolysis to prepare GM3 building block M1, which can avoid multiple chemical procedures. The enzyme is specific for removing the acetyl group at C-6 position from all acetylated monosaccharides. It only needs two steps in high yield to prepare the M1 block from the commercially available D-(+)-mannose. Finally, the 3-*O*-benzoyl-azidosphingosine block was synthesized from commercially available D-(+)-galactose as previous reports with some modifications [20,21].

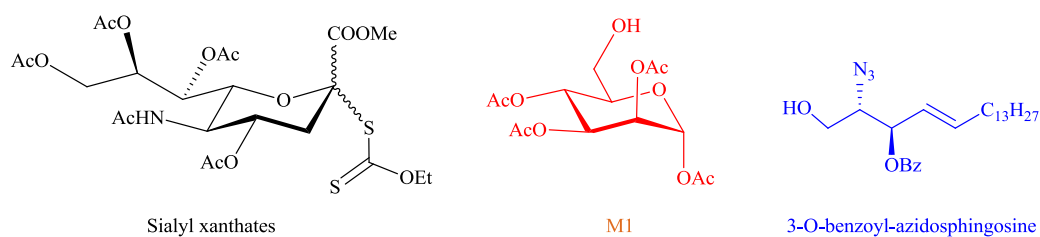
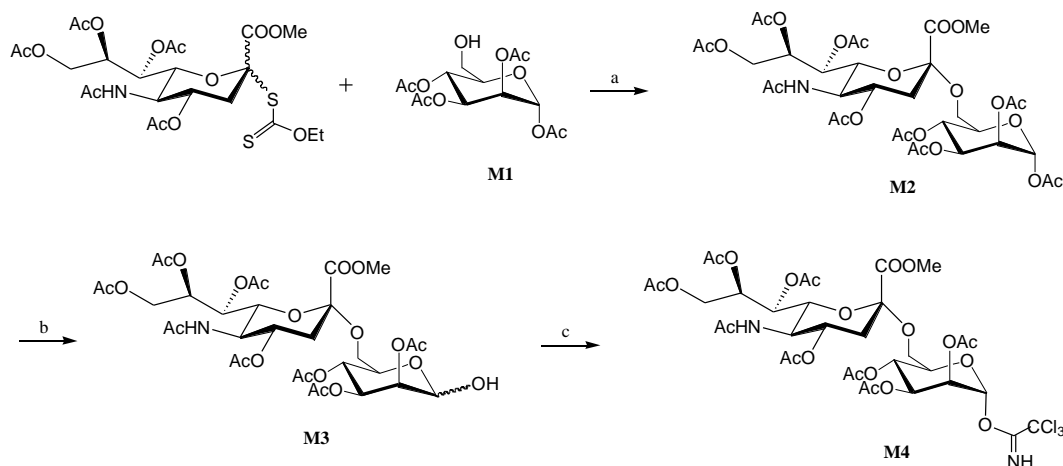


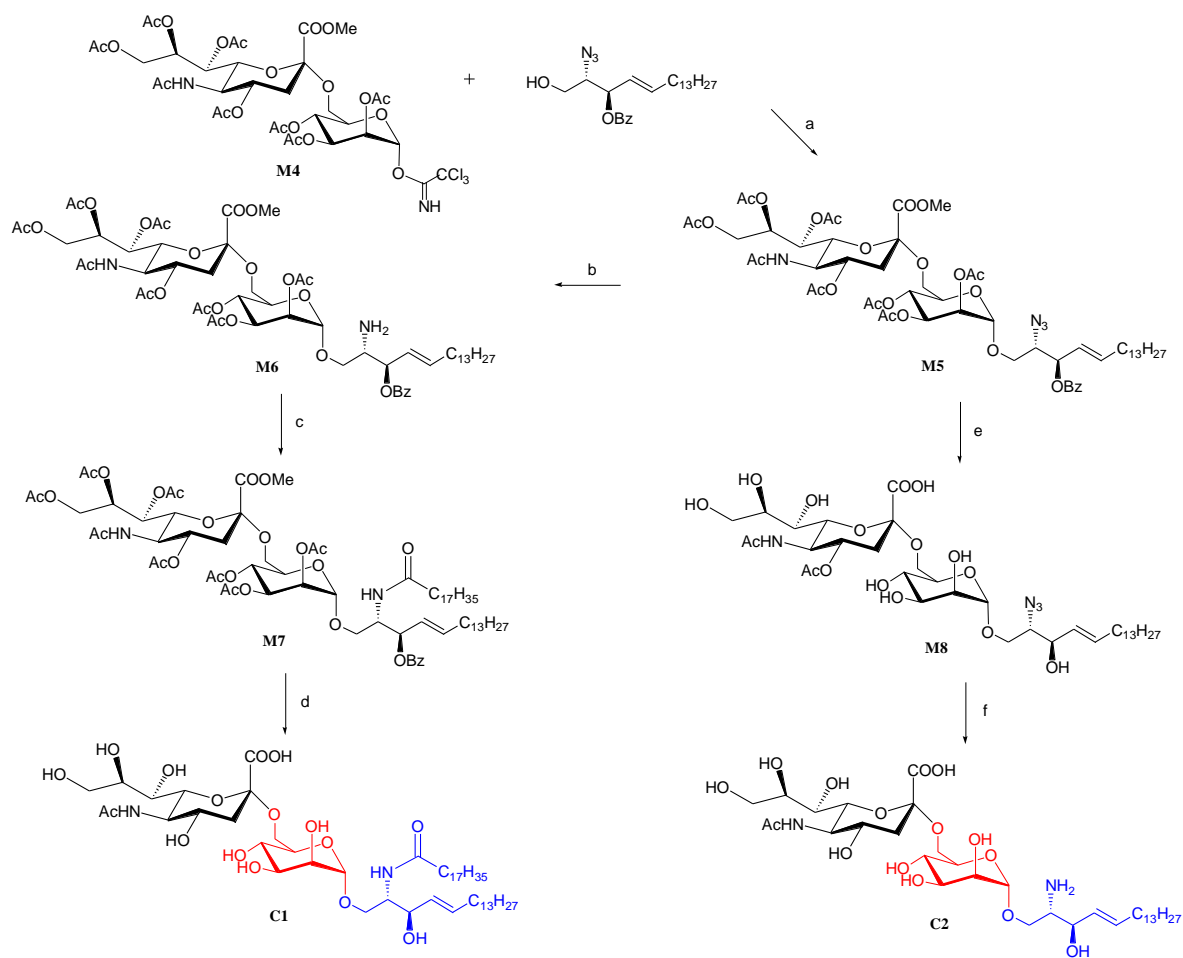
Figure 3. Three key building blocks for synthesizing analogues C1 and C2: sialyl xanthate block, mannose residue M1 block and 3-*O*-benzoyl-azidosphingosine block.

Next, the key step was stereoselective α -sialylation of sialyl xanthates to free 6-OH of all acetylated mannose residue. For this step, Martichonok and Whitesides method was used. At first, sialyl xanthate reacts with PhSOTf to form an oxonium cation. Then the oxonium cation is stabilized by acetonitrile to form a nitrilium cation. The nitrilium cation is known to be involved in an α -sialylation selectivity in acetonitrile solvents [17]. As shown in **Scheme 1**, the α -sialylation reaction was carried out at $-68\text{ }^{\circ}\text{C}$ with benzenesulfonyl chloride (PhSCl) and silver trifluoromethanesulfonate (AgOTf) as promoters in the mixture of $\text{CH}_2\text{Cl}_2:\text{CH}_3\text{CN}$ (1:2) for 3 h. PhSCl was freshly prepared each time. At last, the sialylation product M2 was obtained as α isomer in the yield of 75%. The stereoselectivity of this glycosylation was confirmed by ^1H NMR, for H-3eq of sialic acid residue, the chemical shift was smaller for α -glycosides than for β -glycosides. In this step, it needs the careful chromatography to purify the crude product. Especially, adding 2,6-di-*tert*-butylpyridine (DTBP) to scavenge hydrogen can improve the yield obviously. Then for the α -sialoside M2, the acetyl group at the anomeric center was selectively removed by reaction of PhCH_2NH_2 in the dry THF at RT for 5 h to provide the hemiacetal M3 as an α and β mixture as shown in **Scheme 1**. This crude product M3 was not separated at this stage and was used directly for the next step. The hemiacetal was then treated with trichloroacetonitrile in the presence of 1,8-diazabicyclo [5,4,0] undec-7-ene (DBU) to give the trichloroacetimidate M4 in 70% yield for two steps as shown in **Scheme 1**.



Scheme 1. Reagents and conditions: (a) CH_3CN , CH_2Cl_2 , molecular sieves, r.t., 1 h, AgOTf, DTBP, $-68\text{ }^\circ\text{C}$, PhSCl, 3 h, 75%; (b) PhCH_2NH_2 , THF, r.t., 5 h; (c) CCl_3CN , DBU, CH_2Cl_2 , $-5\text{ }^\circ\text{C}$, 3 h, 70% (two steps from M2).

Glycosylation of 3-*O*-benzoyl-azidosphingosine with trichloroacetimidate M4 was performed by $\text{BF}_3 \cdot \text{Et}_2\text{O}$ as promoter to provide the desired glycolipid M5 in 60% yield, as shown in **Scheme 2**, and the α configuration of the newly introduced glycosidic linkage was obtained due to the axial bond of C-2 position of mannose and neighboring participation effect of C-2 acetyl group. Then the azide group of M5 was reduced by triphenylphosphine in a mixture of toluene and water at $50\text{ }^\circ\text{C}$ for 12 h to give an amino free derivative M6. Again, M6 was not purified and directly reacted with stearic acid in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) in the dry CH_2Cl_2 to give the glycosylated ceramide M7 in 62% yield for two steps from M5. At last, all the acetyl groups of compound M7 were removed by Zemplén transesterification (NaOMe/MeOH). After adding several drops of water, compound C1 was obtained in 95% yield. On the other hand, the acetyl groups of compound M5 were also removed by Zemplén transesterification. After adding several drops of water, product M8 was obtained. Subsequently, without further purification, the azide group in M8 was reduced with propanedithiol/triethylamine to afford the new analogue C2 in 80% yield.



Scheme 2. Reagents and conditions: (a) $\text{BF}_3\text{-Et}_2\text{O}$, CH_2Cl_2 , molecular sieves, $-15\text{ }^\circ\text{C}$, 2.5 h, 60%; (b) PPh_3 , toluene, H_2O , $50\text{ }^\circ\text{C}$, 12 h; (c) Stearic acid, EDC, CH_2Cl_2 , r.t., 20 h, 62% (two steps from M5); (d) NaOMe , MeOH , r.t., 14 h; H_2O , $0\text{ }^\circ\text{C}$, 2 h, 95%; (e) NaOMe , MeOH , r.t., 14 h; H_2O , $0\text{ }^\circ\text{C}$, 2 h; (f) $\text{HS}(\text{CH}_2)_2\text{SH}$, Et_3N , MeOH , r.t., 4 days, 80% (two steps from M5).

2.2. Biological evaluation

2.2.1. GM3 analogues displayed antiproliferative effect on cancer cells

The effect of GM3 analogues C1, C2, C3 and C4 on proliferation of human colon cancer HCT116 cells and chronic myeloid leukemia K562 cells was evaluated by MTT assay after treating with different concentration of GM3 analogues for 48 hours (**Figure 4**). The IC_{50} of analogue C1 for HCT116 and K562 were lower than that of C3, indicated that C1 had more potent antiproliferative activity than C3. Similarly, the IC_{50} of analogue C2 for K562 was lower than C4, therefore C2 possessed stronger cytotoxicity than C4. The results showed that mannose containing analogues C1, C2 could have more potent antiproliferative activity than galactose containing analogues C3 and C4 which we synthesized previously, this observation seemingly implied the cytotoxicity of GM3 analogue may be depended on its

containing sugar residue. The mechanism of antiproliferative activity for exogenous GM3 analogues remains unclear. It has been reported that ganglioside GM3 stimulated tumor suppressor PTEN expression, thus inhibiting HCT116 cell growth [22,23]. Whether the antiproliferative mechanism of our synthetic analogues is also related to PTEN needs further study.

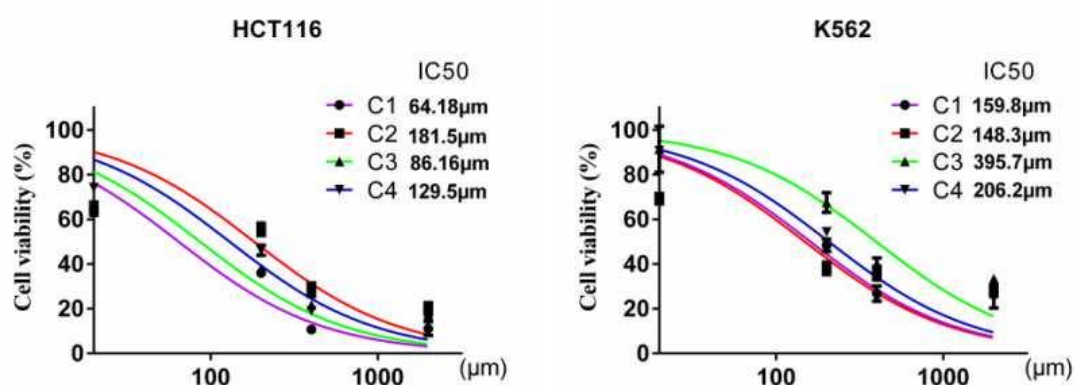


Figure 4. GM3 analogues inhibited cancer cell growth: HCT116 and K562 cells were treated with 0-1000 μM GM3 analogues (C1-C4) for 48 h. Cell viability was measured with MTT and expressed relative to vehicle-treated control. Results are presented as means \pm SD of three independent experiments.

2.2.2. GM3 analogues inhibited cancer cell migration

Cell migration was investigated by a wound healing assay *in vitro*, in which cells migrate bidirectionally from the edges of a scratch wound, and the effect of GM3 analogues on tumor cell migration was expressed as percentage of scratch wound healing. We found that GM3 analogues could inhibit the migration of B16, B16-F10 and HCCLM3 cells (**Figure 5**), and the concentration of GM3 analogues on migration inhibition was 6.25 μM ~ 50 μM , much lower than their antiproliferative concentration, suggesting that compared with inhibiting tumor cell proliferation, the analogues have a stronger effect on tumor cell migration. **Figure 5A** and **Figure 5B** revealed that GM3 analogues at concentration of 6.25 μM and 12.5 μM resulted in a suppressive effects on migration of murine melanoma cells, as if the anti-migration activity of analogues C2 and C4 was stronger than that of C1 and C3. However, except for murine melanoma, whether GM3 analogues also had similar effect on human cancer cell migration. Hence we used the highly metastatic liver cancer cell HCCLM3 to

observe their effect on cell migration, and the results showed that only GM3 analogues C2 and C4 at high concentration of 50 μm produced inhibition of migration for HCCLM3 cells, whereas GM3 analogues C1 and C3 had no effect on cell migration even at 50 μm concentration (**Figure 5C**).

These results showed that analogues C2 and C4 containing sphingosine could have more potent migration inhibitory activity than analogues C1 and C3 containing ceramide, seemingly implied that the anti-migration activity of GM3 analogues may be depended on its containing sphingosine or ceramide. Little is known on the mechanisms which ganglioside GM3 inhibits tumor cell motility. Previous studies suggest that the suppressive effect of ganglioside GM3 on cell motility and growth associating with modulating functions of other molecules co-localized in membrane microdomains, these signal transducer molecules including c-Src, RhoA, H-Ras, FAK, integrin, GFRs and so on, in which GM3 enhances interaction of CD9 with integrin $\alpha 3$ and suppresses c-Src activation following induced integrin activation, and GM2/GM3 complex binds with CD82, subsequently inhibits HGF induced activation of c-Met [24,25]. Whether the mechanism of our compounds involving to these molecules needs further study.

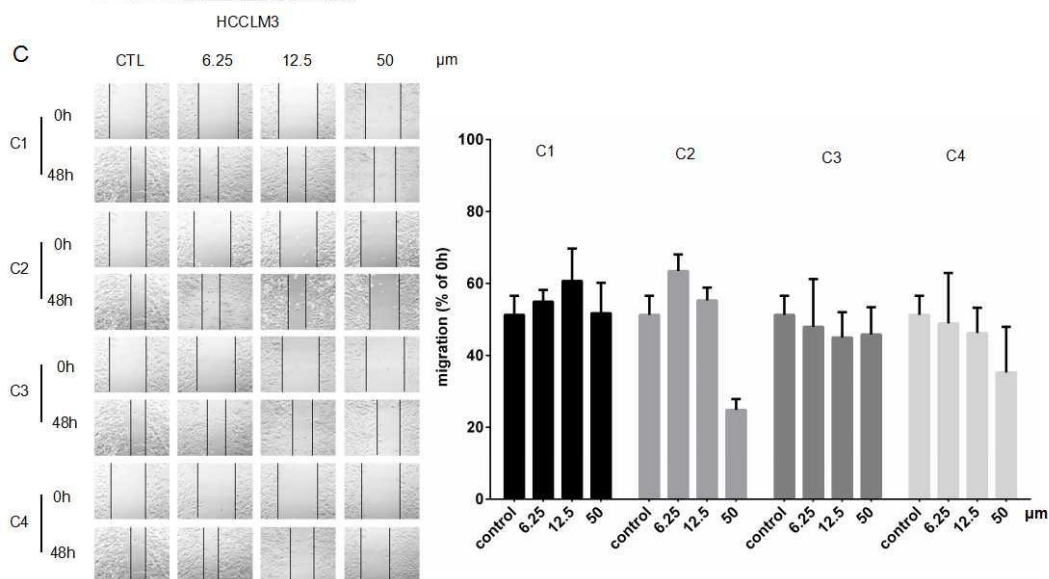
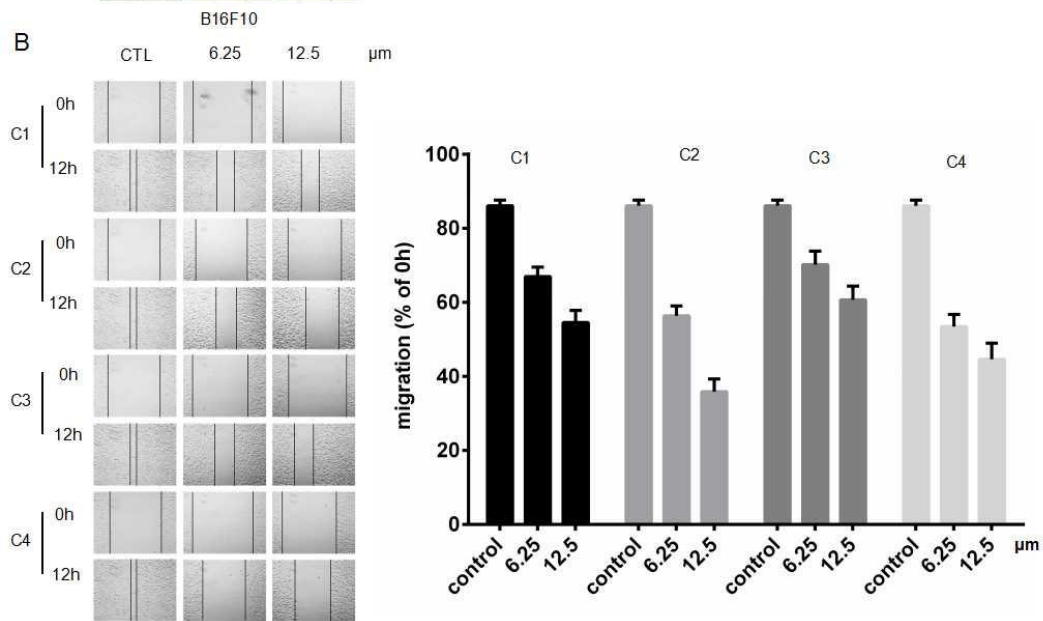
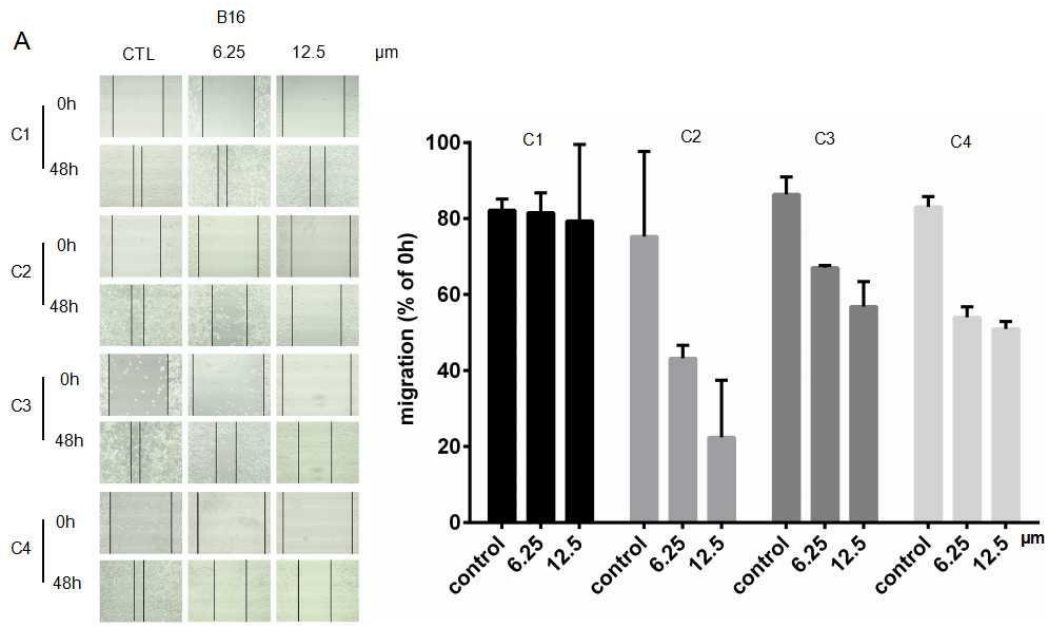


Figure 5. GM3 analogues inhibited cancer cell migration: (A) Effect of GM3 analogues on B16 cell migration after 48 h of exposure. (B) Effect of GM3 analogues on B16-F10 cell migration after 12 h of exposure. (C) Effect of GM3 analogues on HCCLM3 cell migration after 48 h of exposure. Significant inhibition in migration of cancer cells was observed upon treatment with GM3 analogues. Values were calculated as a percentage of the 0 hour (wound width at exposure time point/wound width at 0 h %).

3. Conclusion

In the present study, we successfully synthesized novel mannose containing GM3 analogues by enzymatic hydrolysis and chemical procedures from commercially available substrates and reagents, then their cytotoxicity and anti-migration against cancer cells were evaluated. The synthesized GM3 analogues displayed antiproliferative effect on K562 and HCT116 cancer cells, in which mannose containing analogues C1, C2 could have more potent antiproliferative activity than galactose containing analogues C3 and C4. Our data also showed that some analogues have anti-migration activity on B16, B16-F10 and HCCLM3 cancer cells, particularly these analogues displayed significant anti-migration activity on B16, B16-F10 cancer cells, and the analogues C2 and C4 containing sphingosine could have more potent migration inhibitory activity than analogues C1 and C3 containing ceramide. Our synthesized GM3 analogues exhibit antitumor activities, which provides valuable sights to search new antitumor agents for cancer therapy.

4. Experimental section

4.1. General methods

All chemicals were purchased as reagent grade and used without further purification. All reactions were carried out under N₂ atmosphere and anhydrous conditions with freshly distilled solvents, unless otherwise noted. Reactions were monitored by thin-layer chromatography (TLC) on a precoated plate of silica gel 60 F254 (Merck) and detection by charring with sulfuric acid. Solvents were evaporated under reduced pressure and below 40 °C (water bath). Column chromatography was performed on silica gel 60 (230-400 mesh, Merck). All the new compounds were fully characterized by ¹H and ¹³C NMR, as well as HRMS. ¹H NMR and ¹³C NMR spectra

were recorded at 400 MHz with Bruker AVANCE DRX 400 spectrometer. The chemical shifts were referenced to the solvent peak, 7.26 ppm (^1H) and 77.16 ppm (^{13}C) for CDCl_3 , 3.31 ppm (^1H) and 49.00 ppm (^{13}C) for CD_3OD at 25 °C, and coupling constants were given in Hz. High-resolution mass spectra (HRMS) were recorded with a Bruker microTOF spectrometer in electrospray ionization (ESI) mode, using Tuning-Mix as reference. Optical rotations were measured at 589 nm (Na line) at 20 °C with a Perkin Elmer Model 343 digital polarimeter, using a 10 cm, 1 mL cell.

4.2. *O* - (Methyl 5 - acetamido - 4,7,8,9 - tetra - *O* - acetyl - 3,5 - dideoxy - *D* - glycerol - α - *D* - galactopyranosylate) - (2 \rightarrow 6) - 1,2,3,4 - tetra - *O* - acetyl - α - *D* - mannopyranoside (M2)

A mixture of sialyl xanthates (1.29 g, 2.12 mmol) and compound M1 (500 mg, 1.44 mmol) with 4Å powdered molecular sieves (2.8 g) were dissolved in the dry CH_3CN (30 mL) and CH_2Cl_2 (15 mL) stirring at RT for 1 h. Further, AgOTf (610 mg, 2.34 mmol) and DTBP (0.56 mL, 2.50 mmol) were added, and the mixture was cooled to -68 °C and kept protected from light. Then PhSCl (0.29 mL, 2.50 mmol) in the dry CH_2Cl_2 (1.5 mL) was added by running the solution down the cold wall of the reaction flask. Finally, the mixture was stirred for 2.5 h at -68 °C. After that, the mixture was diluted with a suspension of silica gel (7.5 g) in EtOAc (45 mL), filtered through celite, washed with saturated aqueous NaHCO_3 and water, dried with MgSO_4 , and concentrated under reduced pressure. The residue was chromatographed (Cy-EtOAc 1:3) to give the compound M2 as a white foam (883.9 mg, 75%). $R_f = 0.31$ (Cy-EtOAc 1:3, twice). $[\alpha]_D^{20} = -10.8$ (c 1.0 in CHCl_3). ^1H NMR (400 MHz, CDCl_3): δ 6.05 (d, $J=1.8$ Hz, 1H, H-1'), 5.46 (t, $J=9.5$ Hz, 1H, H-3'), 5.34-5.29 (m, 4H, H7, H8, H-5', NH), 5.23-5.21 (m, 1H, H-2'), 4.91-4.85 (m, 1H, H-4), 4.36 (dd, $J=11.3, 2.7$ Hz, 1H, Ha-9), 4.07-3.97 (m, 5H, H-6, Hb-9, H-4', H-5, Ha-6'), 3.79 (s, 3H, COOCH_3), 3.43 (dd, $J=11.2, 2.6$ Hz, 1H, Hb-6'), 2.64 (dd, $J=11.4, 3.7$ Hz, 1H, H-3eq), 2.18 (s, 3H, OAc), 2.15 (s, 6H, 2 \times OAc), 2.14 (s, 3H, OAc), 2.08 (s, 3H, OAc), 2.04 (s, 3H, OAc), 2.03 (s, 3H, OAc), 2.02 (s, 3H, OAc), 1.89 (t, $J=9.8$ Hz, 1H, H-3ax), 1.87 (s, 3H, NAc). ^{13}C NMR (100 MHz, CDCl_3): δ 170.97 (C=O), 170.57

(C=O), 170.17 (C=O), 170.09 (C=O), 170.04 (C=O), 170.02 (C=O), 169.88 (C=O), 168.94 (C=O), 168.16 (C=O), 167.53 (C=O), 98.42 (C-2), 90.74 (C-1'), 72.31, 71.13, 69.22, 69.09, 68.56, 67.99, 67.21, 65.39 (C-4, C-6, C-7, C-8, C-2', C-3', C-4', C-5'), 62.62 (C-9), 62.37 (C-6'), 52.84 (COOCH₃), 49.41 (C-5), 37.66 (C-3), 23.21 (CH₃, NAc), 21.13 (CH₃, OAc), 20.91 (CH₃, OAc), 20.83 (CH₃, OAc), 20.82 (CH₃, OAc), 20.78 (CH₃, OAc), 20.71 (CH₃, OAc), 20.69 (CH₃, OAc), 20.67 (CH₃, OAc). ESI-HRMS (*m/z*) calcd for C₃₄H₄₇NO₂₂Na [M+Na]⁺: 844.2487, found: 844.2495.

4.3. *O* - (Methyl 5 - acetamido - 4,7,8,9 - tetra - *O* - acetyl - 3,5 - dideoxy - *D* - glycerol - α - *D* - galactopyranosylate) - (2 \rightarrow 6) - 2,3,4 - tri - *O* - acetyl - α - *D* - mannopyranosyl trichloroacetimidate (M4)

The solution of compound M2 (610 mg, 0.74 mmol) in 3 mL of dry THF was added PhCH₂NH₂ (122 μ L, 1.12 mmol) and the mixture was stirred at RT for 5 h. The solvent was evaporated and the residue was taken into CH₂Cl₂ and washed progressively with 1.0 M HCl, saturated aqueous NaHCO₃ and water. The organic layer was dried over MgSO₄ and concentrated, obtained the crude intermediate M3, R_f = 0.29 (EtOAc). The crude intermediate was directly engaged in the following step without further purification. Then the intermediate M3 was dissolved in the dry CH₂Cl₂ (19.6 mL), and 2 mL of trichloroacetonitrile was added to the solution. After that, 192 μ L of DBU was added dropwise at -5 °C, and the mixture was stirred at -5 °C for 3 h. After concentration, the residue was purified by column chromatography (Cy-EtOAc 1:4) to yield compound M4 as pale yellow foam (514 mg, 70% for two steps from M2). R_f = 0.33 (EtOAc). [α]_D²⁰ = +2.8 (*c* 1.0 in CHCl₃). ¹H NMR (400 MHz, CDCl₃): δ 8.74 (s, 1H, NH), 6.25 (d, *J*=1.9 Hz, 1H, H-1'), 5.47 (t, *J*=11.8 Hz, 1H, H-3'), 5.42 (m, 1H, H-5'), 5.36 (dd, *J*=10.2, 3.6 Hz, 1H, H-2'), 5.32-5.29 (m, 3H, H-7, H-8, NH), 4.91-4.85 (m, 1H, H-4), 4.26 (dd, *J*=11.3, 3.2 Hz, 1H, Ha-9), 4.05-3.93 (m, 5H, H-5, H-6, Hb-9, H-4', Ha-6'), 3.78 (s, 3H, COOCH₃), 3.47 (dd, *J*=11.4, 1.9 Hz, 1H, Hb-6'), 2.60 (dd, *J*=11.4, 4.7 Hz, 1H, H-3eq), 2.19 (s, 3H, OAc), 2.14 (s, 3H, OAc), 2.12 (s, 3H, OAc), 2.07 (s, 3H, OAc), 2.03 (s, 3H, OAc), 2.02 (s, 3H, OAc), 1.99 (s, 3H, OAc), 1.94 (t, *J*=9.8 Hz, 1H, H-3ax), 1.86 (s, 3H, NAc). ¹³C NMR (100 MHz, CDCl₃): δ 170.79 (C=O), 170.39 (C=O), 170.02 (C=O), 169.88 (C=O), 169.77 (C=O), 169.74 (C=O), 169.62 (C=O), 168.82 (C=O), 167.48 (C=O), 159.63 (C=NH), 98.23 (C-2), 94.65 (C-1'), 90.38 (CCl₃), 72.20, 71.69, 69.04, 68.88,

68.03, 67.78, 67.06, 65.05 (C-4, C-6, C-7, C-8, C-2', C-3', C-4', C-5'), 62.46 (C-9), 62.13 (C-6'), 52.64 (COOCH₃), 49.24 (C-5), 37.47 (C-3), 23.02 (CH₃, NAc), 20.92 (CH₃, OAc), 20.86 (CH₃, OAc), 20.67 (CH₃, OAc), 20.65 (CH₃, OAc), 20.62 (CH₃, OAc), 20.54 (CH₃, OAc), 20.46 (CH₃, OAc). ESI-HRMS (*m/z*) calcd for C₃₅H₄₈N₂Cl₃O₂₁Na [M+Na]⁺: 960.1713, found: 960.1704.

4.4. *O*-(Methyl 5 - acetamido - 4,7,8,9 - tetra - *O* - acetyl - 3,5 - dideoxy - *D* - glycerol - *D* - galactose - 2 - nonulopyranosylate) - (2 → 6) - (2,3,4 - tri - *O* - acetyl - α - *D* - mannopyranosyl) - (1 → 1) - (2*S*, 3*R*, 4*E*) - 2 - azido - 3 - *O* - benzoyl - 4 - octadecene - 1,3 - diol (M5)

The compound M4 (229 mg, 0.25 mmol) and 3-*O*-benzoyl-azidosphingosine (180 mg, 0.42 mmol) in 12 mL of dry CH₂Cl₂ was stirred with 4Å powdered molecular sieves (1 g) at RT for 0.5 h. The mixture was then cooled to -15 °C, and BF₃·Et₂O (156 μ L, 1.24 mmol) was added dropwise, stirred for 2.5 h at -15 °C, and then filtered through celite. The filtrate was washed with saturated aqueous NaHCO₃ and water, dried over MgSO₄ and concentrated under reduced pressure. The residue was applied to a chromatography eluted with Cy-EtOAc 1:5 to give the product M5 (177 mg, 60%) as an amorphous solid. *R*_f = 0.47 (EtOAc). [α]_D²⁰ = -24.5 (*c* 1.0 in CHCl₃). ¹H NMR (400 MHz, CDCl₃): δ 8.06-8.03 (m, 5H, Ar-H), 5.82-5.75 (m, 1H, H-5''), 5.53-5.48 (m, 2H, H-3'', H-4''), 5.38-5.31 (m, 6H, H-4, H-7, H-8, NH, H-2', H-3'), 4.91-4.87 (m, 1H, H-4'), 4.86 (d, *J*=2.0 Hz, 1H, H-1'), 4.36 (m, 1H, Ha-9), 4.28 (dd, *J*=12.4, 2.7 Hz, 1H, Hb-9), 3.98-3.92 (m, 5H, H-5, H-5', H-6', H-2''), 3.86 (dd, *J*=10.4, 3.6 Hz, 1H, Ha-1''), 3.76 (s, 3H, COOCH₃), 3.52-3.44 (m, 2H, H-6, Hb-1''), 2.59 (dd, *J*=12.7, 4.6 Hz, 1H, H-3eq), 2.17-2.15 (m, 2H, H₂-6''), 2.14 (s, 3H, OAc), 2.13 (s, 3H, OAc), 2.11 (s, 3H, OAc), 2.05 (s, 3H, OAc), 2.02 (s, 3H, OAc), 1.99 (s, 3H, OAc), 1.98 (s, 3H, OAc), 1.90 (t, *J*=11.2 Hz, 1H, H-3ax), 1.85 (s, 3H, NAc), 1.39-1.36 (m, 2H, H₂-7''), 1.24 (s, 20H, 10 × CH₂), 0.87 (t, *J*=6.8 Hz, 3H, CH₃). ¹³C NMR (100 MHz, CDCl₃): δ 170.86 (C=O), 170.57 (C=O), 170.22 (C=O), 170.07 (C=O), 169.99 (C=O), 169.86 (C=O), 169.79 (C=O), 169.14 (C=O), 167.83 (C=O), 165.13 (PhC=O), 138.67 (C-5''), 133.29 (C, CH aromatic), 133.25 (C aromatic), 129.81 (C, CH aromatic), 129.78 (C, CH aromatic), 129.76 (C, CH aromatic), 123.29 (C-4''), 98.45 (C-1'), 98.36 (C-2), 74.55 (C-3''), 72.45 (C-6), 69.64 (C-4'), 69.49 (C-3'), 69.19 (C-2'), 69.06 (C-4), 68.22 (C-8), 67.88 (C-1'), 67.29 (C-5'), 66.76 (C-7), 63.81 (C-2''), 62.36 (C-6'), 61.93 (C-9), 52.73 (COOCH₃), 49.37

(C-5), 37.89 (C-3), 32.36 (C-6''), 29.63, 29.58, 29.41, 29.34 (CH₂), 22.68 (CH₃, NAc), 21.09 (CH₃, OAc), 20.89 (CH₃, OAc), 20.85 (CH₃, OAc), 20.79 (CH₃, 2 × OAc), 20.71 (CH₃, 2 × OAc), 20.67 (CH₃, OAc), 14.11 (CH₃). ESI-HRMS (*m/z*) calcd for C₅₇H₈₂N₄O₂₃Na [M+Na]⁺: 1213.5268, found: 1213.5259.

4.5. *O* - (Methyl 5 - acetamido - 4,7,8,9 - tetra - *O* - acetyl - 3,5 - dideoxy - *D* - glycerol - α - *D* - galactopyranosylate) - (2 \rightarrow 6) - (2,3,4 - tri - *O* - acetyl - α - *D* - mannopyranosyl) - (1 \rightarrow 1) - (2*S*, 3*R*, 4*E*) - 2 - octadecanamido - 3 - *O* - benzoyl - 4 - octadecene - 1,3 - diol (M7)

The compound M5 (80 mg, 0.068 mmol) in 7.8 mL of toluene and 0.32 mL of water was added 44.0 mg of triphenylphosphine. The mixture was stirred at 50 °C for 12 h. After concentration, the crude residue M6, R_f = 0.23 (EtOAc), was directly used for the next step. The intermediate M6, stearic acid (66 mg, 0.24 mmol), EDC (56 mg, 0.36 mmol) in 10.8 mL of CH₂Cl₂ were stirred at RT for 20 h. Then the mixture was washed with water, dried over MgSO₄ and concentrated under reduced pressure. The resulting residue was purified by column chromatography (Cy-EtOAc 1:3) to afford compound M7 (76 mg, 62% for two steps from M5) as an amorphous solid. R_f = 0.39 (Cy-EtOAc 1:3). [α]_D²⁰ = -10.5 (*c* 1.0 in CHCl₃). ¹H NMR (400 MHz, CDCl₃): δ 8.02-7.99 (m, 2H, Ar-H), 7.58-7.54 (m, H, Ar-H), 7.45-7.42 (m, 2H, Ar-H), 5.93-5.83 (m, 2H, NH, H-5''), 5.59-5.48 (m, 2H, H-3'', H-4''), 5.34-5.23 (m, 4H, H-8, H-7, H-3', NH), 5.12 (d, *J*=9.3 Hz, 1H, H-5'), 4.94-4.83 (m, 1H, H-2'), 4.73 (d, *J*=1.9 Hz, 1H, H-1'), 4.54-4.47 (m, 1H, H-4), 4.26 (dd, *J*=12.4, 2.6 Hz, 1H, Ha-9), 4.07-4.01 (m, 3H, H-5, H-6, Hb-9), 3.94-3.89 (m, 3H, Ha-6', Hb-6', H-2''), 3.81 (dd, *J*=10.5, 3.1 Hz, 1H, Ha-1''), 3.77 (s, 3H, COOCH₃), 3.66 (dd, *J*=5.7, 2.5 Hz, 1H, H-4'), 3.40 (d, *J*=8.9 Hz, 1H, Hb-1''), 2.59 (dd, *J*=12.8, 4.6 Hz, 1H, H-3eq), 2.25-2.18 (m, 2H, COCH₂), 2.13 (s, 3H, OAc), 2.12 (d, *J*=1.5 Hz, 6H, 2 × OAc), 2.05 (s, 3H, OAc), 2.02 (s, 3H, OAc), 2.01 (s, 3H, OAc), 1.99 (s, 3H, OAc), 1.94 (t, *J*=7.4 Hz, 1H, H-3ax), 1.86 (s, 3H, NAc), 1.64-1.59 (m, 2H, H₂-6''), 1.24 (s, 52H, 26 × CH₂), 0.87 (t, *J*=6.9 Hz, 6H, 2 × CH₃). ¹³C NMR (100 MHz, CDCl₃): δ 173.34 (C=O), 171.41 (C=O), 171.03 (C=O), 170.65 (C=O), 170.54 (C=O), 170.44 (C=O), 170.30 (C=O), 170.27 (C=O), 169.59 (C=O), 168.22 (C=O), 165.73 (PhC=O), 138.22 (C-5''), 133.57 (C, CH aromatic), 130.46 (C aromatic), 130.15 (2C, CH aromatic), 128.90 (2C, CH aromatic), 125.08 (C-4''), 98.98 (C-1'), 98.90 (C-2), 74.51 (C-3''), 72.90 (C-6), 69.96 (C-4'), 69.78 (C-3'), 69.74 (C-2'), 69.47 (C-4), 68.61 (C-8), 67.70 (C-1''), 67.38

(C-5'), 66.60 (C-7), 63.51 (C-6'), 62.80 (C-9), 53.25 (COOCH₃), 51.37 (C-2''), 49.97 (C-5), 37.34 (C-3), 32.38, 30.17, 30.16, 30.12, 29.82 (CH₂), 23.66 (CH₃, NAc), 21.33 (CH₃, OAc), 21.30 (CH₃, OAc), 21.26 (CH₃, 2 × OAc), 21.21 (CH₃, OAc), 21.17 (CH₃, 2 × OAc), 14.57 (2 × CH₃). ESI-HRMS (*m/z*) calcd for C₇₅H₁₁₈N₂O₂₄Na [M+Na]⁺: 1453.7972, found: 1453.7982.

4.6. *O* - (5 - Acetamido - 3,5 - dideoxy - *D* - glycerol - α - *D* - galactopyranosyl - 2 - nonulopyranosylonic acid) - (2 → 6) - (α - *D* - mannopyranosyl) - (1 → 1) - (2*S*, 3*R*, 4*E*) - 2 - octadecanamido - 4 - octadecene - 1,3 - diol (C1)

A solution of compound M7 (60 mg, 0.042 mmol) in 8.2 mL of NaOMe/MeOH (0.04 M) was stirred at RT for 14 h. A few drops of water were added at 0 °C. Then the mixture was stirred at RT for 2 h, and further neutralized by Amberlite IR 120/H⁺ ion exchange resin. After filtration and concentration, the residue obtained was flash-chromatographed, eluting with CHCl₃-MeOH 3:1 to yield the analogue C1 (40 mg, 95%) as a white amorphous solid. R_f = 0.33 (EtOAc-*i*PrOH-H₂O 3:2:1). [α]_D²⁰ = +10.1 (*c* 1.0 in CHCl₃-MeOH 1:1). ¹H NMR (400 MHz, CD₃OD): δ 5.74-5.67 (m, 1H, H-5''), 5.49-5.43 (m, 1H, H-4''), 4.72 (d, *J*=1.8 Hz, 1H, H-1'), 4.09-4.00 (m, 2H, Ha-1'', H-3''), 3.99-3.95 (m, 1H, H-3'), 3.87-3.80 (m, 5H, H-4, H-5, H-4', Ha-6', Hb-1''), 3.70-3.51 (m, 9H, H-6, H-7, H-8, H-9, H-2', H-5', Hb-6', H-2''), 2.71 (dd, *J*=12.1, 4.9 Hz, 1H, H-3eq), 2.18 (t, *J*=6.8 Hz, 2H, CH₂C(O)), 2.06-2.01 (m, 2H, H₂-6''), 2.00 (s, 3H, NAc), 1.79 (t, *J*=12.1 Hz, 1H, H-3ax), 1.58 (m, 2H, CH₂CH₂C(O)), 1.29 (s, 50H, 25 × CH₂), 0.90 (t, *J*=7.4 Hz, 6H, 2 × CH₃). ¹³C NMR (100 MHz, CD₃OD): δ 174.16 (C=O), 169.18 (C=O), 168.65 (C=O), 134.18 (C-5''), 128.44 (C-4''), 100.09 (C-1'), 97.53 (C-2), 75.05 (C-3''), 73.46 (C-3'), 71.06 (C-4'), 70.56 (C-6), 69.81 (C-2'), 69.32 (C-4), 69.18 (C-5'), 68.95 (C-1''), 68.54 (C-8), 68.15 (C-7), 63.93 (C-6'), 63.53 (C-9), 55.01 (C-2''), 50.97 (C-5), 40.12 (C-3), 30.17, 30.16, 30.12, 29.82 (CH₂), 22.71 (CH₃, NAc), 15.47 (2 × CH₃). ESI-HRMS (*m/z*) calcd for C₅₃H₉₇N₂O₁₆ [M-H]⁻: 1017.6916, found: 1017.6912.

4.7. *O* - (5 - Acetamido - 3,5 - dideoxy - *D* - glycerol - α - *D* - galactopyranosyl - 2 - nonulopyranosylonic acid) - (2 → 6) - (α - *D* - mannopyranosyl) - (1 → 1) - (2*S*, 3*R*, 4*E*) - 2 - amino - 4 - octadecene - 1,3 - diol (C2)

The compound M5 (40.0 mg, 0.034 mmol) in 6.7 mL of NaOMe/MeOH (0.04 M) was stirred at RT for 14 h. Then a few drops of water were added at 0 °C. After

stirring at RT for 2 h, the mixture was neutralized by Amberlite IR 120/H⁺ ion exchange resin. After filtration and concentration, the residue was dried in vacuo to afford crude intermediate M8, R_f = 0.43 (EtOAc-*i*PrOH-H₂O 3:2:1). The crude intermediate M8 in the anhydrous MeOH (1.8 mL) were added propane-1,3-dithiol (0.18 mL) and triethylamine (0.18 mL), and the mixture was stirred at RT for 4 days. A white precipitate was formed. After filtration and washing with MeOH, the filtrate was concentrated under reduced pressure. The residue obtained was chromatographed, eluting with CHCl₃-MeOH 3:1 to yield the analogue C2 (20.2 mg, 80% for two steps from M5) as a white amorphous solid. R_f = 0.28 (EtOAc-*i*PrOH-H₂O 3:2:1). [α]_D²⁰ = -3.1 (*c* 1.0 in CHCl₃: MeOH = 1:1). ¹H NMR (400 MHz, CD₃OD): δ 5.90-5.85 (m, 1H, H-5''), 5.53-5.48 (m, 1H, H-4''), 4.76 (d, *J*=1.8 Hz, 1H, H-1'), 4.01-3.78 (m, 8H, H-4, H-5, H-3', H-4', Ha-6', H-1'', H-3''), 3.72-3.6 (m, 7H, H-6, H-8, H-9, H-2', Hb-6', H-2''), 3.52 (dd, *J*=9.1, 1.5 Hz, 1H, H-7), 3.44-3.40 (m, 1H, H-5'), 2.83 (dd, *J*=12.1, 4.9 Hz, 1H, H-3eq), 2.15-2.08 (m, 2H, H₂-6''), 2.00 (s, 3H, NAc), 1.79 (t, *J*=12.1 Hz, 1H, H-3ax), 1.43-1.41 (m, 2H, H-7''), 1.30 (s, 20H, 10 × CH₂), 0.90 (t, *J*=7.4 Hz, 3H, CH₃). ¹³C NMR (100 MHz, CD₃OD): δ 175.56 (C=O), 175.53 (C=O), 136.32 (C-5''), 128.71 (C-4''), 102.59 (C-1'), 101.46 (C-2), 74.42, 73.48, 72.96, 72.42, 71.66, 71.35, 71.20, 70.30, 69.45, 68.33 (C-4, C-6, C-7, C-8, C-2', C-3', C-4', C-5', C-1'', C-3''), 64.74 (C-6'), 64.51 (C-9), 58.46 (C-2''), 54.20 (C-5), 42.45 (C-3), 33.06, 30.81, 30.78, 30.73, 30.45 (CH₂), 23.72 (CH₃, NAc), 14.42 (CH₃). ESI-HRMS (*m/z*) calcd for C₃₅H₆₃N₂O₁₅ [M-H]: 751.4307, found: 751.4315.

4.8. Biological evaluation

4.8.1. Cell culture

Human colon cancer HCT116 cells, human leukemia K562 cells, highly metastatic human liver cancer HCCLM3 cells, murine melanoma B16 cells and highly metastatic murine melanoma cells B16-F10 cells were originally obtained from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (China), and cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone), as well as penicillin and streptomycin (100 mg/ml each), at 37 °C in a humidified atmosphere of 5% CO₂.

4.8.2. Cytotoxicity assay

Cell viability was assessed using MTT method. Briefly, the cells (8×10^3 /well) were seeded into 96-well plates, and were treated with GM3 analogues (C1, C2, C3, C4) at 20, 200, 400, 1000 μ M or vehicle DMSO for 48 h. At the end of the incubation period, 20 μ l (5 mg/mL) MTT solution was added into each well of the 96-well plate. Following 4 h of incubation at 37 °C and 5% CO₂, absorbance was detected at a wavelength of 570 nm. The results are presented as means \pm SD from the three independent experiments.

4.8.3. Wound healing test

We used wound healing test to evaluate the effect of GM3 analogues on tumor cell motility by B16, B16-F10 and HCCLM3 tumor cell lines [26]. The cells (1.5×10^5 cells/24 wells) were cultured into 24-well plates and grown in RPMI-1640 medium containing 10% FBS to nearly confluent cell monolayer. A 10 μ L plastic pipette tip was used to draw a linear “wound” in the cell monolayer of each well. The monolayer was then washed twice with serum free RPMI-1640 medium to remove debris or detached cells, and GM3 analogues were added at different concentrations in fresh medium with FBS, RPMI-1640 medium containing DMSO was added to the control well as the solvent control, and subsequently cultured for 12 h or 48 h depending on the different ability of the cell migration. The wound healing of the scratched cells was photographed under a DMIL LED AE2000 inverted microscope (Leica, Wetzlar, Germany) and the effect of GM3 analogues on tumor cell motility was expressed as migration % of 0 hour (wound width at exposure time point/wound width at 0 hour). The experiments were performed in triplicate.

Acknowledgments

We thank the China Scholarship Council (CSC) for Ph.D. fellowships to Changping ZHENG and Zhihao LI. Financial supports from the Centre National de la Recherche Scientifique (CNRS) and the Sorbonne Université in France, Joint Funds for the

Innovation of Science and Technology, Fujian province, China (2016Y9059), and the Projects of Industry-Academy Cooperation for Science and Technology of Fujian Province, China (2016Y4005) are gratefully acknowledged.

Supporting information

Supplementary data of NMR spectra associated with this article can be found in the online version.

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