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Effect of lipid mimetics of GM3 and lyso-GM3 dimer on EGF receptor tyrosine kinase and EGF-induced signal transduction

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Abstract

The tyrosine kinase activity associated with epidermal growth factor receptor (EGFR) has been a target in studies of pharmacological reagents to inhibit growth of cancer cells, which are mostly of epidermal origin. Lyso-GM3 dimer showed stronger inhibitory effect on the tyrosine kinase of EGFR than GM3, with minimal cytotoxicity (Y. Murozuka, *et al.*, *Glycoconj. J.*, 24: 551–63, 2007). Synthesis of lipids with sphingosine requires many steps, and the yield is low. Biocombinatory approach overcame this difficulty; however, products required a C₁₂ aliphatic chain, rather than the sphingosine head group (Y. Murozuka, *et al.*, *Chem. Biodivers.* 2: 1063–78, 2005). The present study was to clarify the effects of these lipid mimetics of GM3 and lyso-GM3 dimer on EGFR tyrosine kinase activity, and consequent changes of the A431 cell phenotype, as follows. (i) A lipid mimetic of lyso-GM3 dimer showed similar strong inhibitory effect on EGF-induced EGFR tyrosine kinase activity, and similar low cytotoxicity, as the authentic lyso-GM3 dimer. (ii) A lipid mimetic of lyso-GM3 dimer inhibited tyrosine phosphorylation of EGFR or its dimer to a level similar to that of the authentic lyso-GM3 dimer, but more strongly than GM3 or a lipid mimetic of GM3. (iii) Associated with the inhibitory effect of a lipid mimetic of lyso-GM3 dimer on EGF-induced EGFR kinase activity, only Akt kinase activity was significantly inhibited, but kinases associated with other signal transducers were not affected. (iv) The cell cycle of A431 cells, and the effects of GM3 and lipid mimetic of lyso-GM3 dimer, were studied by flow cytometry, measuring the rate of DNA synthesis with propidium iodide. Fetal bovine serum greatly enhanced S phase and G₂/M phase. Enhanced G₂/M phase was selectively inhibited by pre-incubation of A431 cells with a lipid mimetic of lyso-GM3 dimer, whereas GM3 had only a minimal effect.

Keywords

DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; sf, serum-free

1. Introduction

Ganglioside GM3 has been known to inhibit tyrosine kinase associated with epidermal growth factor receptor (EGFR) [1]. Lyso-GM3 was detected as a minor component of A431 cells, and was found to inhibit EGF-induced tyrosine phosphorylation of EGFR much more strongly than GM3 [2]. Since lyso-GM3 is cytotoxic, we studied lyso-GM3 derivatives retaining inhibitory

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effect on receptor tyrosine kinase but displaying lower cytotoxicity, and found that two sialyllactosyl sphingosine (lyso-GM3) residues conjugated through the 2-amino group of sphingosine to glutaminyllutamine (lyso-GM3 dimer) satisfied these criteria [3]. However, synthesis of sphingosine and sphingolipids (GM3, lyso-GM3, and lyso-GM3 dimer) requires many steps, and it is difficult to achieve satisfactory yields. To overcome this difficulty, a biocombinatory system using B16 melanoma cells was employed in which exogenous lactosyl-2-azido-dodecanol was converted efficiently to sialyllactosyl-2-azido-dodecanol, which was then converted to various lipid mimetics of GM3, lyso-GM3, and lyso-GM3 dimer. This first step sialylation process through melanoma cells is only efficient when aliphatic chain length is limited to C₁₂, *i.e.*, a dodecanol derivative [4].

The purpose of the present study is to determine whether these lipid mimetics of GM3, lyso-GM3, and lyso-GM3 dimer, which lack sphingosine, display biological effects similar to those of authentic GM3, lyso-GM3, and lyso-GM3 dimer which possess sphingosine [3], *i.e.*, 2-amino-1,3-dihydroxy-(*trans*-4,5)-octadecene with a *D*-erythro geometric isomer at C1-3 (2*S*, 3*R*). The biological effects of lipid mimetics of GM3 and lyso-GM3 dimer were studied by incubating them with A431 cells, stimulating the incubated cells with EGF, and observing effect on (i) tyrosine kinase activity of EGFR; (ii) EGFR dimerization; (iii) Akt and MAPK activation; (iv) changes in the cell cycle. These EGF-induced phenotypic changes of A431 cells were clearly inhibited by a lipid mimetic of lyso-GM3 dimer, to a similar degree as with authentic lyso-GM3 dimer.

2. Materials and methods

2.1 Synthesis of lipid mimetics of lyso-GM3 or of GM3

2.1.1 General methods, and chemicals used in synthesis—¹H and ¹³C NMR spectra were recorded on a 600 MHz JEOL spectrometer. Chemical shifts were assigned using COSY and HMQC. All reactions were monitored by thin layer chromatography (TLC) on Silica Gel 60 F-254 (E. Merck), with detection by UV light or by visualizing with spraying with anisaldehyde-H₂SO₄ or the phosphomolybdic acid reagent. Column chromatography was performed on Silica Gel 60 (70–230 mesh, E. Merck) or flash Silica Gel. The ESI-MS spectrum was recorded on a Bruker Esquire Ion Trap Mass Spectrometer.

Sodium methoxide and triphenylphosphine were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and stearic acid, boron trifluoride etherate and 4-(*N,N*-dimethylamino)pyridine (DMAP) were from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC), *N,N*-diisopropylethylamine (DIEA) and benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate were obtained from Sigma-Aldrich (Milwaukee, WI). Sialyllactose was kindly donated by Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan).

2.1.2 Synthesis of 2-azido-1-dodecanol, lipid mimetic of lyso-GM3, or of GM3—2-azido-1-dodecanol was synthesized as described previously [4]. Fully O-acetylated sialyllactose, linked to 2-amino-dodecanol (structure 3 in scheme 1 of Fig. 1) was synthesized as described [4,5]. Stearic acid was added to the amino group of this compound (structure 4 in scheme 2 of Fig. 1), followed by de-acetylation to yield a ceramide mimetic of GM3 (structure 5 in scheme 2 of Fig. 1), as described previously [6–8]. See figure legends for reaction conditions and yields. ESI-MS: calcd. for C₅₃H₉₈N₂O₂₀ (M + H)⁺, 1083.7; found (M + H)⁺, 1083.8.

2.1.3 Synthesis of a lipid mimetic of lyso-GM3 dimer—The compound (structure 7 in scheme 3 of Fig. 1) was synthesized as follows. Fmoc-(Glu)₂-NH₂ (11.3 mg, 22.7 μmol), PyBOP (24.8 mg, 47.7 μmol), DMAP (cat.) and DIEA (15.4 μl, 90.8 μmol) were dissolved in

anhydrous DMF (1 ml) and the mixture was stirred at room temperature under argon for 30 min. Then, the mixture was added dropwise to a solution of compound **3** (67.4 mg, 54.5 μ mol) in DMF (3 ml) and stirred at room temperature under argon for 16 h. Toluene was added to the mixture and evaporated. The product was purified by flash silica gel column chromatography (chloroform: methanol = 10:1 as eluent) to give compound **6**: 61.7 mg, 92.6%; structure **6** in scheme 3 of Fig. 1 (61.0 mg, 20.8 μ mol) was dissolved in methanol (3 ml), and 24.3 mg (450 μ mol) of sodium methoxide was added. The reaction mixture was stirred at room temperature for overnight and neutralized with a small portion of Amberlite IR120B NA, H⁺ form. After removal of resin by filtration, the methanol solution was evaporated to dryness. The obtained syrup was dissolved in distilled water, washed with CHCl₃ and then lyophilized, which gave a white-cotton-like material (structure **7** in scheme 3 of Fig. 1): 26.0 mg, 66.8%. ¹H-NMR (600 MHz, methanol-*d*₃): δ 8.21 (m, 2H, -NHAc), 7.70 (m, 2H, -NHCO-), 4.41 (m, 2H, H-1'), 4.28 (m, 2H, H-1), 2.82 (m, 2H, H-3eq'), 1.82 (m, 2H, H-3ax'), 1.61-1.26 (m, -CH₂-), 0.87 (t, 6H, *J* = 7.2 Hz, -CH₃). ESI-MS: calcd. for C₈₀H₁₄₁N₇O₄₂ (M + H)⁺, 1872.9; found (M + H)⁺, 1873.1.

2.2 Biological effects of a lipid mimetic of GM3, or of a lyso-GM3 dimer, as compared to the authentic sphingolipids

2.2.1 Antibodies and reagents—Mouse anti-EGFR mAb, mouse anti- γ -tubulin mAb and ribonuclease A were from Sigma, and mouse anti-pTyr mAb (PY20) was from BD Transduction Laboratories. Anti-MAPK (p44/42) rabbit IgG, anti-phospho-p44/42 MAPK (Thr202/Tyr204) rabbit IgG, anti-Akt rabbit IgG, and anti-phospho-Akt (Ser473)(193H12) rabbit IgG were from Cell Signaling Technologies (Beverly, MA). Goat anti-mouse IgG labeled with horseradish peroxidase was from Southern Biotechnology (Birmingham, AL), and goat anti-rabbit IgG-HRP were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-GM3 IgG₃ mAb DH2 was prepared as described [9]. Ganglioside GM3 was purchased from Matreya (Pleasant Gap, PA). EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride), a Micro-BCA kit and the SuperSignal West Pico Chemiluminescent Substrate were from Pierce (Rockford, IL). Propidium iodide was from Fluka/ Sigma-Aldrich.

2.2.2 Cell culture—Human epidermoid carcinoma A431 cells [10,11] were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT) as described previously [1], but with addition of 1 mM sodium pyruvate, 100 IU/ml penicillin, and 10 μ g/ml streptomycin (Mediatech, Inc, Herndon, VA) at 37°C, 5% CO₂.

2.2.3 Inhibition of EGFR phosphorylation in cultured A431 cells—Aliquots of CM 2:1 solution containing various quantities of GM3, a lipid mimetic of GM3 (structure **5** in Scheme 2 of Fig. 1), a lyso-GM3 dimer, or a lipid mimetic of lyso-GM3 dimer (structure **7** in Scheme 3 of Fig. 1) were dried under N₂, mixed with ethanol, dried again, and sonicated in serum-free (sf) DMEM at indicated concentrations (μ M). A431 cells were seeded (1 $\times$ 10⁵ cells/well) and cultured in a 24-well plate in DMEM containing 5% fetal bovine serum for 48 h, and in sf DMEM for 24 h prior to confluence. Then, cells were washed twice with sf DMEM, incubated in 200 μ l of sf DMEM containing various concentrations of four compounds (GM3, a lipid mimetic of GM3, a lyso-GM3 dimer, or a lipid mimetic of lyso-GM3 dimer) for 24 h at 37°C. After incubation, the medium was removed and 200 μ l of 100 ng/ml hEGF in sf DMEM was added to the well and incubated for 1 h at 4°C. Cells were then washed and lysed in 200 μ l of Triton RIPA buffer (1% Triton X-100, 150 mM NaCl, 25 mM Tris, pH 7.5, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM pyrophosphate, 50 mM NaF) containing 1 mM Na₃VO₄, 75 units of aprotinin and 2 mM phenylmethanesulfonyl fluoride at 4°C. The lysate was centrifuged at 15,000 rpm for 15 min at 4°C, and the supernatant was subjected to 7% SDS-PAGE and Western blot after determination of protein concentration. The intensity of

bands on the Western blot was determined by densitometry using the Scion image program. The membrane was re-blotted with γ -tubulin antibody, and the amount of protein was adjusted by γ -tubulin levels. Densitometry results were normalized with controls.

2.2.4 Cytotoxic effects—Cytotoxic effects of GM3, a lyso-GM3 dimer, and their lipid mimetics on A431 cells were tested by the following method. Cells (1×10^5) were seeded and cultured in 48-well plate in DMEM containing 5% FBS for 24 h, and in sf DMEM for 24 h, until confluence. Then, cells were washed twice with sf DMEM, incubated in 100 μ l sf DMEM containing various concentrations of GM3, the lyso-GM3 dimer, or their lipid mimetics, for 24 h at 37°C. After incubation, cells were washed with PBS and harvested with trypsin-EDTA, centrifuged, and suspended in 100 μ l PBS. The number of living cells, excluding those stained by Trypan Blue, was counted. Viability was expressed as described in the Fig. 3B legend.

2.2.5 Preparation of A431 cell membranes—A431 cells were grown in 8 culture dishes (diameter 150 mm; Nunc, Rochester, NY) in DMEM with 10% FBS until they reached confluence. Cells were scraped from plates, washed in PBS(-), and homogenized by 50 strokes of a Dounce homogenizer using a tight-fitting pestle (Wheaton Scientific, Millville, NJ) at 4°C in 7 ml of homogenizing buffer (20 mM HEPES buffer, pH 7.4, containing 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF, and 10 μ g/ml leupeptin). The lysate was centrifuged at $1,500 \times g$ for 10 min at 4°C, and the supernatant was further centrifuged at $30,000 \times g$ for 60 min at 4°C. The pellet was rehomogenized with a small Dounce homogenizer in 0.5 ml of ice-cold 20 mM MES buffer (pH 7.0, containing 100 μ g/ml leupeptin at a protein concentration of 10 mg/ml), aliquoted, and stored at -80°C until use.

2.2.6 Effects of GM3, a lipid mimetic of GM3, a lyso-GM3 dimer, and a lipid mimetic of lyso-GM3 dimer, on EGFR dimerization in vitro—Aliquots of CM 2:1 solution containing GM3, a lipid mimetic of GM3 (compound **5**), a lyso-GM3 dimer, or a lipid mimetic of lyso-GM3 (compound **7**) were dried under N_2 , mixed with ethanol, dried again, and sonicated in 20 mM MES buffer (pH 7.0). 4 μ l aliquots of A431 membrane (40 μ g protein) and 4 μ l of each sample were mixed, and incubated for 15 min at 22°C. Next, 2 μ l of 10 μ g/ml EGF in MES buffer (EGF¹) was added to each tube and incubated for 45 min at 22°C. Incubation was continued for 30 min in the absence or presence of 2 μ l of 5 mg/ml EDC at 30°C. Under these conditions, final concentrations of these samples are indicated in the figure legends. Membranes were washed twice with 200 μ l of cold 20 mM HEPES buffer (pH 7.4) containing 100 μ g/ml leupeptin, and recovered by centrifugation at $15,000 \times g$ for 10 min at 4°C. The membrane pellet was solubilized for 30 min at 22°C in 4 μ l of 20 mM HEPES buffer (pH 7.4) containing 10% glycerol, 100 μ g/ml leupeptin, and 0.5% Triton X-100. The lysates were mixed with 4 μ l of 20 mM HEPES buffer (pH 7.4). 4 μ l aliquots of membrane samples were incubated with 4 μ l of 10 μ g/ml EGF in HEPES buffer (EGF²), 4 μ l of 20 mM HEPES buffer (pH 7.4), 2 μ l of 10 mM $MnCl_2$, 2 μ l of 100 μ M $ZnCl_2$, and 2 μ l of 500 μ M Na_3VO_4 for 10 min at 22°C, then phosphorylated for 10 min at 4°C with 2 μ l of 50 μ M ATP. Final concentrations of $MnCl_2$, $ZnCl_2$, and Na_3VO_4 in the incubation mixture were 1 mM, 10 μ M, and 50 μ M, respectively. The reaction was stopped by addition of 20 μ l of 2x sample buffer, and samples were heated for 5 min at 95°C. They were applied to 4% SDS-PAGE and subjected to Western blotting.

2.2.7 Effects of GM3 and a lipid mimetic of lyso-GM3 dimer on signal transducers—A431 cells were seeded and cultured in a 24-well plate as above. After pre-incubation, cells were washed twice with sf DMEM, incubated in 200 μ l of sf DMEM containing 100 μ M of GM3 or a lipid mimetic of lyso-GM3 dimer (compound **7**), for 24 h at 37°C. After incubation, medium was removed and 200 μ l of 100 ng/ml hEGF in sf DMEM was added to the well and incubated for 1 h at 4°C. Cells as above were subjected to 7% SDS-PAGE and Western blot

after determination of protein concentration. The intensity of the Western blot of bands corresponding to EGFR, Akt, and MAPK by their respective antibodies, and the activated form (*i.e.*, anti-phosphotyrosine PY20 to EGFR, anti-phospho-p44/42 MAPK (Thr202/Tyr204), and anti-phospho-Akt (Ser473) (193H12)) was determined by densitometry using the Scion image program.

For determining dose-dependent inhibition, A431 cells were prepared the same as above, and treated with various concentration of lipid mimetic of lyso-GM3 dimer.

2.2.8 Effects of lipid mimetic of lyso-GM3 dimer on cell cycle—A431 cells were seeded (1×10^5 cells/well) and cultured in a 24-well plate in DMEM containing 5% FBS for 48 h, and in sf DMEM for 24 h. Then, cells were washed twice with sf DMEM, incubated in 500 μ l of sf DMEM in the absence or presence of 100 μ M of GM3 or lipid mimetic of lyso-GM3 dimer (compound **7**), for 24 h at 37°C. After incubation, the medium was removed and 1 ml of 10% FBS DMEM was added to the well and the plate was further incubated for 48 h (cells incubated with sf DMEM were used as negative control). The cell cycle pattern, based on DNA cytograms, was determined by flow cytometry of cells pre-incubated with GM3 or with a lipid mimetic of lyso-GM3 dimer as above. The basic procedure was as described previously, *e.g.*, [12]; Beckman Coulter flow cytometry information, Application Note 2, <http://www.bc-cytometry.com>. For this procedure, cells were washed with PBS(-) and harvested by trypsinization, washed in ice-cold PBS, and fixed in 70% ethanol for 2 h. The fixed cells were washed in ice-cold PBS and treated with 1 μ g/ml of propidium iodide (PI) with 250 μ g/ml RNase A in PBS(-) for 30 min at 37°C in the dark. After incubation, cells were analyzed by flow cytometry using a Coulter EPICS XL flow cytometer (Beckman Coulter, Fullerton, CA). From each sample, 20,000 events were analyzed. Cell doublets were gated out by examining PI integral intensity and PI peak intensity.

3. Results

3.1 Thin layer chromatography of GM3, a lyso-GM3 dimer, and their lipid mimetics

TLC behavior of four synthetic compounds, GM3, a lyso-GM3 dimer, a lipid mimetic of GM3, and a lipid mimetic of lyso-GM3 dimer, which were tested for effect on EGFR tyrosine kinase, EGFR dimerization, and EGF-induced signal transduction, is shown in Fig. 2. The lyso-GM3 dimer was clearly detected by TLC immunostaining with anti-GM3 mAb DH2, whereas the lipid mimetic of lyso-GM3 dimer (sialyllactosyl-2-amino-dodecanol) was not reactive with DH2. The lipid mimetic of GM3 showed slightly lower migration and slightly lower DH2 reactivity than GM3.

3.2 Inhibitory effect of GM3, a lyso-GM3 dimer, and a lipid mimetics of GM3 and of lyso-GM3 dimer, on EGFR tyrosine kinase activity of A431 cells in culture

The effect of various quantities of four compounds as described above on EGFR tyrosine kinase activity of A431 cells was determined as described in “Materials and methods”, by intensity of Western blot bands of EGFR with anti-tyrosine phosphate antibodies and with anti-EGFR antibodies. GM3 did not inhibit kinase activity at 5–10 μ M, and inhibition was minimal even at 50–200 μ M (Fig. 3A, **group 1**). A lipid mimetic of GM3 (**group 2**), and of a lyso-GM3 dimer (**group 3**), displayed a similar degree of inhibition at 50–100 μ M. In contrast, a lipid mimetic of lyso-GM3 dimer displayed clear dose-dependent inhibition (**group 4**).

The cytotoxic effect on A431 cells of various doses of these compounds was determined by Trypan Blue exclusion test, as shown in Fig. 3B. Lyso-GM3 dimer at 100–200 μ M displayed significant cytotoxicity (**group 3**), whereas lipid mimetic of lyso-GM3 dimer showed lower cytotoxicity, particularly at 200 μ M (**group 4**).

3.3 Effects of GM3, a lipid mimetic of GM3, a lyso-GM3 dimer, and a lipid mimetic of lyso-GM3 dimer, on EGFR dimerization in a membrane fraction of A431 cells *in vitro*

A431 cell membranes were prepared as described in Sec. 2.2.5. Aliquots of membrane were incubated respectively with four compounds as above with concentration 50, 100, or 200 μ M, followed by initial addition of EGF ("EGF¹") to stimulate EGFR tyrosine kinase *in vitro*. EGFR dimer formed by activation was stabilized by the coupling reagent EDC. The recovered protein was incubated with ATP and a second addition of EGF ("EGF²"), followed by SDS-PAGE and Western blot with mAbs to EGFR and to tyrosine phosphate (PY20), as described in Sec. 2.2.8 (Fig. 4). The procedure is systematically expressed in the table at the bottom of Fig. 4A.

Dose-dependent inhibitory effects of GM3 (Fig. 4B), a lipid mimetic of GM3 (Fig. 4C), a lyso-GM3 dimer (Fig. 4D), and a lipid mimetic of lyso-GM3 dimer (Fig. 4E) on tyrosine phosphorylation, defined by PY20 reactivity with EGFR monomer as a percent of the entire PY20 reactivity with the monomer (170 kDa) plus the dimer (340 kDa), were determined. All four compounds mentioned above showed an inhibitory effect on tyrosine phosphorylation of both mono- and dimeric EGFR. Interestingly, there appeared to be no difference in inhibitory effects on receptor phosphorylation among these compounds.

3.4 Effects of GM3, and a lipid mimetic of lyso-GM3 dimer, on EGF-induced activation of EGFR Tyr kinase, Akt Ser kinase, and MAPK Thr/ Tyr kinase

A431 cells were seeded, grown to near confluency, washed with sf DMEM, incubated with the same medium containing 100 μ M GM3 or a lipid mimetic of lyso-GM3 dimer, washed, and stimulated with EGF in sf DMEM for 1 hr. Aliquots of lysate were analyzed by Western blot with antibodies directed to various receptors, signal transducers (Akt, MAPK), or their activated (phosphorylated) form, as described in "Materials and methods". Intensities of bands are summarized in Fig. 5A, and densitometry by Scion image is shown in Fig. 5B. As shown in Fig. 5, phosphorylation of Akt was inhibited in relation to the degree of inhibition of EGFR kinase activity. However, unexpectedly, MAPK signaling was not affected by either GM3 or a lipid mimetic of lyso-GM3 dimer.

We further determined the dose-dependent effects of lipid mimetic of lyso-GM3 dimer on phosphorylation of EGFR, Akt and MAPK (Fig. 6) to confirm these observations. Whereas EGFR and Akt kinase activities were inhibited in a dose-dependent manner, phosphorylation of MAPK was not affected even at high concentration.

3.5 Effect on the cell cycle of A431 cells by GM3, and by a lipid mimetic of lyso-GM3 dimer

A431 cells were seeded, cultured, incubated in sf DMEM for 24 h, then pre-incubated with GM3 or a lipid mimetic of lyso-GM3 dimer, washed, trypsinized, fixed in ethanol, and treated with 1 μ g/ml of propidium iodide with RNase A in PBS(-) for determination of cell cycle status by flow cytometry, as described in Sec. 2.2.9. Cell populations at G₀/G₁ phase (2N), S phase, and G₂/M phase (4N) were calculated (as %), as shown in the table associated with Fig. 7. Flow cytometric patterns of A431 cells stimulated by FBS showed significant enhancement of the S phase and G₂/M phase population, as indicated by enhanced DNA synthesis probed by propidium iodide labeling (Fig. 7B vs. Fig. 7A). Enhanced G₂/M phase induced by FBS stimulation was strongly inhibited and G₀/G₁ phase was arrested when cells were pre-incubated with a lipid mimetic of lyso-GM3 dimer (Fig. 7D vs. 7B). Pre-incubation with GM3 had minimal effect on cell cycle (Fig. 7C vs. 7B).

Discussion

Glycosphingolipids, including gangliosides, have the novel capability to interact with many functional membrane proteins, such as cytoplasmic signal transducers (Src family kinases,

small G-proteins) [13,14], tetraspanins (CD9, CD81, CD82) [15–18], adhesion receptors (integrins) [19,20], and growth factor receptors [1,21–23]. Studies on modulatory effects, initially observed with GM3 on EGFR tyrosine kinase [1], have been extended to effects of GM1, GD1a, GD1b, and GT1b on platelet-derived growth factor receptor [21,24], GM1 and plasmalopsychosine on nerve growth factor receptor [23,25], and GM3 and sialyl-nLc4 on insulin receptors [22,26]. Therefore, such glycosphingolipid effects can be considered as common in growth factor and hormone receptors, in general (for review see [27,28]).

EGFR tyrosine kinase is regarded as an important target to find effective pharmacological reagents to inhibit growth and invasiveness of many cancer cells, since the majority of human cancers are derived from epidermal tissue, and enhanced tumor cell growth and invasiveness are controlled by enhanced EGFR tyrosine kinase. Previously, various drugs showing inhibitory effect on tyrosine phosphorylation of specific cytoplasmic site of EGFR have been studied, including Phase 1 clinical trials. Unfortunately, these reagents displayed cytotoxic effects, and are undergoing further clinical studies [29–32].

Therefore, different approaches, such as that based on the effect of lyso-GM3, are of interest. This compound, originally found as a minor component of A431 cells displayed a much stronger inhibitory effect than GM3 on EGFR tyrosine kinase *in vitro* in isolated membranes, although lyso-GM3 displayed strong cytotoxicity. Lyso-GM3 dimer showed inhibitory effect similar to that of lyso-GM3 but had no cytotoxic effect [18]. Therefore, lyso-GM3 dimer, or its mimetics, are interesting candidates for tumor growth suppression. Difficulty in large-scale production of such sphingolipids, which involves multi-step synthesis of sialyllactosyl-sphingosine as the basic unit, was circumvented by sialylation of lactosyl-2-amino-dodecanol, efficiently performed using B16 melanoma cells in culture. The products through biocombinatory synthesis have a common sialyllactosyl structure as authentic GM3, lyso-GM3, or the lyso-GM3 dimer, but the lipid moiety contains 2-amino-dodecanol instead of sphingosine. These products are termed "lipid mimetics" of GM3, lyso-GM3, or the lyso-GM3 dimer. Use of sialylation of lactosyl-2-amino-dodecanol limits the aliphatic chain length to C₁₂, since B16 melanoma cells are incapable of taking up lactosyl-2-aminododecanol, converting it to sialyl derivatives, and releasing it from the cell when a mimetic with a longer chain aglycon is used.

It is essential to compare inhibitory effects of lipid mimetics of compounds on EGFR tyrosine kinase and various consequent phenotypic responses of A431 cells, to those of authentic sphingolipid compounds. It should be noted that reactivity of the sialyllactosyl group linked to the 1-hydroxy group of sphingosine is different from that of the same glycosyl group linked to the 1-hydroxy group of 2-amino-dodecanol, since lipid mimetics of lyso-GM3 dimer did not react with mAb DH2, whereas authentic lyso-GM3 dimer showed clear reactivity (Fig. 2). It was therefore expected that the inhibitory effect of authentic lyso-GM3 dimer on EGFR tyrosine kinase would differ from that of the lipid mimetic of lyso-GM3 dimer. However, contrary to this expectation, results of the present study indicate that the lipid mimetic of lyso-GM3 dimer and authentic lyso-GM3 dimer showed similar inhibitory effects. *I.e.*, EGFR tyrosine kinase does not discriminate between the authentic structures *vs.* the lipid mimetic of lyso-GM3 dimer. The former has a C₁-C₅ structure of D(+)-erythro(2*S*,3*R*)-2-amino-1,3-dihydroxy(*trans*-4,5)octadecene, while the latter has a simple 2-amino aliphatic chain.

The mechanism of EGF-induced activation of EGFR tyrosine kinase is based on the occurrence of receptor-to-receptor interaction, according to the Schlessinger-Ullrich hypothesis [33]. The inhibitory effect of GM3 on such activation is assumed to involve blocking of receptor-to-receptor interaction. Studies along this line indicate that receptor dimer formation is not strongly blocked by GM3, but tyrosine phosphorylation on both monomeric and dimeric EGFR is inhibited by GM3 [34,35]. In these studies, A431 cells, or membrane EGFR, and the

compound to be tested were first incubated, and then EGF-induced dimerization of EGFR was measured. In this case, the dose-dependent inhibitory effect on tyrosine phosphorylation in both monomeric and dimeric EGFR was observed. In the present study, a similar dose-dependent inhibitory effect of the lipid mimetic of GM3 and the lipid mimetic of lyso-GM3 dimer, on both monomeric and dimeric EGFR, was observed (Fig. 4). In contrast, when EGFR was initially activated to induce dimerization, followed by incubation with the compounds to be tested, it appeared that the compounds inhibited tyrosine phosphorylation regardless of the occurrence of dimerization, *i.e.*, the compound may inhibit tyrosine phosphorylation without involvement of receptor dimerization. Further studies on this mechanism are planned.

While it is clear that EGF-induced activation of EGFR tyrosine kinase is inhibited by lipid mimetic of the lyso-GM3 dimer, to the same extent as by the authentic lyso-GM3 dimer, the question arises: which signaling molecules are affected by the change in EGFR tyrosine kinase? In many previous studies, signaling through receptor tyrosine kinase led to MAPK through SOS, Ras GTP, and MAPKKK (Raf), *e.g.*, [36]; for review see [37]. Our results indicate, unexpectedly, that MAPK kinase activity was not affected following EGF-induced activation of A431 cells, and that a lipid mimetic of lyso-GM3 dimer did not affect MAPK kinase. It is possible, however, that MAPK kinase in liver may be deactivated rapidly by a specific tyrosine phosphatase (MKP-1) acting on MAPK, *e.g.*, [38,39]. In addition, cSrc determined with Src tyrosine kinase at Tyr416, Csk kinase affecting Src tyrosine at 527, and phosphoinositide-3 kinase (PI3K), were not affected by EGF stimulation of A431 cells. The only kinase activated by EGF, and whose activity was inhibited by pre-incubation of A431 cells with the lipid mimetic of lyso-GM3 dimer, was Akt kinase.

The inhibitory effect of authentic lyso-GM3 dimer on EGF-induced A431 cell proliferation was suggested by inhibition of thymidine incorporation in our previous study [3]. Possible cell growth inhibition by the lipid mimetic of lyso-GM3 dimer, but not by GM3, was indicated by its inhibitory effect on the FBS-induced G₂/M phase in A431 cells (Fig. 7). It is well documented that inhibition of the EGFR-dependent pathway induces a perturbation of cell cycle progression and notably G₁ arrest. GM3 was incapable of inhibiting FBS-induced enhancement of the G₂/M phase. These observations, taken together, suggest that the lipid mimetic of lyso-GM3 dimer inhibits EGFR tyrosine kinase, leading to inhibition of mitogenesis of A431 cells. The actual link between EGFR tyrosine kinase and nuclear DNA synthesis, leading to mitogenesis, and the mechanism of the effect of authentic lyso-GM3 dimer and the lipid mimetic of lyso-GM3 dimer, remain to be elucidated.

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Abbreviations and glossary

Lipid mimetic of GM3, GM3 ganglioside with ceramide-like lipid lacking sphingosine.; Lyso-GM3, sialyllactosyl sphingosine.; Lipid mimetic of lyso-GM3 (LM-GM3), sialyllactosyl-2-

amino-dodecanol.; Lipid mimetic of lyso-GM3 dimer (LM-dimer), sialyllactosyl-2-amino-dodecanol linked together through glutamic acid dimer..

Scheme 1

Scheme 2

Scheme 3

Fig. 1.

Schemes for synthesis of a lipid mimetic of GM3, lyso-GM3, and a lyso-GM3 dimer
Scheme 1. Synthesis of lipid mimetic of lyso-GM3. Reagents and conditions: (a) Ac₂O, pyridine, DMF, 50 °C, 24 h, 83.7%; (b) 2-azido-1-dodecanol, BF₃·(C₂H₅)₂O, molecular sieves, 1,2-dichloroethane, 40 °C, 24 h, 31.7%; (c) PPh₃, H₂O, DMF, 50 °C, 20h, 91.6%.

Scheme 2. Synthesis of lipid mimetic of GM3 (LM-GM3). Reagents and conditions: (a) compound 3 was reacted with stearic acid in EDC, DMAP, anhyd. DMF, at room temperature (r.t) for 17 h; the yield of compound 4, 67.9%; (b) deacetylated in NaOMe, MeOH, r.t., 16 h, the yield of compound 5 (*i.e.*, 2-N-stearylaminododecyl-O-(5-acetamido- α -3,5-dideoxy-D-glycero-D-galacto-2-nonulopyranosylonic acid)-(2 $\rightarrow$ 3)-O- β -D-galactopyranosyl-(1 $\rightarrow$ 4)- β -D-glucopyranoside), 55.8%.

Scheme 3. Assembly of a lipid mimetic of lyso-GM3 with a glutamic acid dimer (synthesis of a lipid mimetic of lyso-GM3 dimer; LM-dimer). Reagents and conditions: (a) compound 3 was reacted with Fmoc-(Glu)₂-NH₂ in PyBOP, DIEA, DMAP, anhyd. DMF, r.t, 16 h, the yield of compound 6, 92.6%; (b) NaOMe, MeOH, r.t., 14 h, the yield of compound 7, 66.8%. Details of conditions: see Sec. 2.1.3.

Abbreviations: DIEA, N,N-diisopropylethylamine; DMAP, 4-(N,N-dimethylamino)pyridine; DMF, dimethylformamide; EDC, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride.

Fig. 2.

HPTLC of authentic GM3, a lipid mimetic of GM3, authentic lyso-GM3 dimer, and a lipid mimetic of lyso-GM3 dimer, developed with chloroform/methanol/ 0.2% CaCl_2 in H_2O (50:40:10, v/v/v). **(A)** Detection by spraying with 0.2% orcinol in 2 M aqueous sulfuric acid, heated for 15 min at 120 °C. **(B)** Immunostaining with mAb DH2. TLC plate was fixed by 90 sec immersion in 0.5% polyisobutyl-methacrylate solution in a mixture of chloroform/ hexane 1:9, dried, blocked with 1% BSA and goat serum (1:50) in PBS for 1 hr at r.t., and reacted overnight with mAb DH2 (hybridoma supernatant) at 4 °C. The plate was washed with PBS, then incubated with HRP-conjugated secondary antibody for 1 hr, followed by detection with SuperSignal substrate.

Lane 1: authentic GM3 ($R_f=0.38$). **Lane 2:** lipid mimetic of GM3 ($R_f=0.36$). **Lane 3:** authentic lyso-GM3 dimer ($R_f=0.18$). **Lane 4:** lipid mimetic of lyso-GM3 dimer ($R_f=0.17$).

Fig. 3.

Inhibitory effects of GM3, a lipid mimetic of GM3, a lyso-GM3 dimer, and a lipid mimetic of lyso-GM3 dimer, on EGFR tyrosine kinase of A431 cells, and their cytotoxicities

Panel A: A431 cells were seeded and cultured in DMEM with serum to near-confluency, washed with and incubated for 24 hr in sf medium, followed by incubation with 200 μl sf DMEM with 0, 5, 10, 50, 100, or 200 μM (concentration means sialyllactose concentration) of each of the four compounds. Cells in each well were stimulated by EGF followed by preparation of lysate, and subjected to SDS-PAGE with Western blot, using a pair of mAbs, one to EGFR, the other to tyrosine phosphate. Inhibitory effects of various compounds on EGFR tyrosine kinase were expressed in terms of the ratio of a Western blot band defined by

anti-pTyr mAb (PY20) / anti-EGFR mAb (ordinate). Abscissa: numbers indicate final concentrations of sialyllactose units (μM). GM3, authentic GM3. LM-GM3, a lipid mimetic of GM3. Lyso-GM3 dimer, authentic lyso-GM3 dimer. LM-dimer, a lipid mimetic of lyso-GM3 dimer.

Panel B: Cytotoxic effects of various quantities of compounds on A431 cells.

Fig. 4.

Effect of GM3, a lipid mimetic of GM3, a lyso-GM3 dimer, and a lipid mimetic of lyso-GM3 dimer, on EGFR dimerization *in vitro*

Panel A: Upper: Western blot analysis of the monomer and dimer of phospho-EGFR. Analyzed in triplicate. The membrane was re-blotted with γ -tubulin antibody. Lane 1: control (EGF¹-, EGF²+), lane 2: control (EGF¹+, EGF²+), lane 3: GM3 50 μ M, lane 4: GM3 100 μ M, lane 5: GM3 200 μ M, lane 6: lipid mimetic of GM3 50 μ M, lane 7: lipid mimetic of GM3 100 μ M, lane 8: lipid mimetic of GM3 200 μ M, lane 9: lyso-GM3 dimer 50 μ M, lane 10: lyso-GM3 dimer 100 μ M, lane 11: lyso-GM3 dimer 200 μ M, lane 12: lipid mimetic of lyso-GM3 dimer 50 μ M, lane 13: lipid mimetic of lyso-GM3 dimer 100 μ M, lane 14: lipid mimetic of lyso-

GM3 dimer 200 μ M. Concentrations represent sialyllactose units. Lower: γ -tubulin loading control. Abbreviations are as in Fig. 3A.

Table attached to Fig. 4A: A procedure for determining the effect of four compounds on EGF-induced tyrosine phosphorylation associated with EGFR monomer vs. dimer. The order of procedure is schematically shown from top to bottom (see “Materials and methods”, Sec. 2.2.5 and 2.2.6).

Panel B–E: Scion image densitometry analysis of EGFR-monomer and EGFR-dimer bands incubated with authentic GM3, LM-GM3, lyso-GM3 dimer and LM-dimer, normalized with control.

Fig. 5.

Effect of GM3 and a lipid mimetic of lyso-GM3 dimer on EGFR tyrosine kinase, Akt kinase, and MAPK.

Panel A: Effect of GM3 and a lipid mimetic of lyso-GM3 dimer on EGFR, Akt, MAPK, and their phosphorylation patterns (P-EGFR, P-Akt, P-MAPK) induced by EGF in A431 cells.

These bands were determined by Western blot analysis using a sequential procedure as described in Sec. 2.2.7. γ -tubulin as loading control. **Lane 1:** no pre-incubation with GSL or lipid mimetic; no EGF stimulation. **Lane 2:** same as lane 1, but with EGF stimulation. **Lane 3:** pre-incubation with 100 μ M GM3, with EGF stimulation. **Lane 4:** pre-incubation with 100 μ M of a lipid mimetic of lyso-GM3 dimer, with EGF stimulation.

Panel B: Intensity of bands for P-EGFR/ EGFR (top), P-Akt/ Akt (middle), and P-MAPK/ MAPK (bottom) from Panel A, determined by densitometry using the Scion Image program. **Column 1:** no pre-incubation with GSL or lipid mimetic; no EGF stimulation. **Column 2:** same as 1, but with EGF stimulation. **Column 3:** pre-incubation with 100 μ M GM3, with EGF stimulation. **Column 4:** pre-incubation with 100 μ M lipid mimetic of lyso-GM3 dimer, with EGF stimulation.

Fig. 6.

Dose-depend inhibitory effect of lipid mimetic of lyso-GM3 dimer, on EGFR tyrosine kinase, Akt kinase, and MAPK.

Panel A: Effect of the lipid mimetic of lyso-GM3 dimer on EGFR, Akt, MAPK, and their phosphorylation patterns (P-EGFR, P-Akt, P-MAPK) induced by EGF in A431 cells. These bands were determined by Western blot analysis using the sequential procedure described in Sec. 2.2.7. **Lane 1:** untreated; no EGF stimulation. **Lane 2:** same as lane 1, but with EGF stimulation. **Lane 3:** pre-incubation with 5 μ M LM-dimer, with EGF stimulation. **Lane 4:** 10 μ M LM-dimer. **Lane 5:** 50 μ M LM-dimer. **Lane 6:** 100 μ M LM-dimer. **Lane 7:** 200 μ M LM-dimer.

Panel B: Intensity of bands for P-EGFR/ EGFR (top), P-Akt/ Akt (middle), and P--MAPK/ MAPK (bottom) from Panel A, determined by densitometry using the Scion Image program.

	A	B	C	D
FBS	–	+	+	+
GM3	–	–	+	–
LM-dimer	–	–	–	+

	G_0/G_1 (%)	S (%)	G_2/M (%)
control (–)	85.9	5.1	9.0
control (+)	71.5	13.3	15.2
GM3	76.0	8.7	15.3
LM-dimer	83.4	6.8	9.8

Fig. 7.

Effect of GM3 and a lipid mimetic of lyso-GM3 dimer on the A431 cell cycle, determined as described in Sec. 2.2.8. Phases G_0/G_1 , S, and G_2/M are indicated by a flow cytometric pattern of DNA synthesis probed by propidium iodide, and changes of the pattern induced by FBS stimulation with or without pre-incubation with GM3 or a lipid mimetic of lyso-GM3 dimer, are shown in Panels **A**, **B**, **C**, **D**. Combinations of treatment for each panel are shown at the top of the figure. **A**: control (no lipid pre-incubation) without FBS stimulation. **B**: control (no lipid pre-incubation) with FBS stimulation. **C**: GM3 pre-incubation, with FBS stimulation. **D**: lipid mimetic of lyso-GM3 dimer pre-incubation, with FBS stimulation. % of phases G_0/G_1 , S, and G_2/M were calculated as shown in the table.