In vivo anti-tumour effect of 3'-sulphonoquinovosyl 1'-monoacylglyceride isolated from sea urchin (Stronglylocentrotus intermedius) intestine

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Summary Extracts from sea urchin intestine were screened for anti-tumour drugs. Four glycolipids, 3'-sulphonoquinovosyl-1', 2'-diacylglyceride (A-4), 3'-sulphononoquinovosyl-1'-monoacylglyceride (2'-lyso A-4, A-5), NeuGcα2-6Glβ1-1ceramide (A-6) and HSO3-8NeuGcα2-6Glβ1-1ceramide (A-7), were isolated from the intestine of sea urchin, Stronglylocentrotus intermedius, and characterized by means of proton nuclear magnetic resonance spectroscopy and fast atom bombardment mass spectrometry. When tested for cytotoxic activity against tumour cells in vitro, A-5 showed significant activity, but A-4, -6 and -7 did not. In addition, the hydrophilic derivatives of A-4 or -5 had no cytotoxicity. Furthermore, the anti-tumour effects on nude mice bearing solid tumours of a human lung adenocarcinoma cell line A-549 were evaluated in vivo using A-4 and -5. As a result, A-5 was found to be significantly effective in suppressing the growth of solid tumours, whereas A-4 had no effect. Pathologically, the solid tumours showed haemorrhagic necrosis areas after treatment with A-5. In this study, we have demonstrated the anti-tumour effect of sulphonoquinovosyl-lysoglyceride (A-5), which provides important information that this sulpholipid could be a useful drug for cancer chemotherapy.

Keywords: sulpholipid; anti-tumour; cancer chemotherapy; sea urchin

Since the growth and drug resistance characteristics of neoplastic tissues are rich in diversity, it is important to search for many new sources of cancer chemotherapy drugs (Riordom and Ling, 1985; Tsuru, 1988; Bishop, 1994; Hartwell and Kastan, 1994; Rabbitts, 1994). Recently, marine invertebrates have shown particular promise as a new source of anti-tumour drugs. Through evolution, as their physical defences are poor, they have developed chemical arsenals to defend themselves from various enemies. Therefore, many researchers have been investigating toxic substances produced by marine invertebrates that could be applicable for destroying tumours. The successful extractions of many anti-tumour substances, such as didemin (Venditti, 1983), bryostatin (Pettit et al, 1982) and dolastatin (Pettit et al, 1987) derived from marine invertebrates, have been reported and have reached clinical trial. In our research, we have employed the sea urchin intestine as a drug source and screened for glycolipids having anti-tumour activity.

Glycolipids play an important role in cell membranes. It is well known that changes in the quality and density of gangliosides are observed with tumorigenesis (Ravindranath et al, 1991; Jennemann et al, 1990). In addition, tyrosine phosphorylation of the epidermal growth factor receptor is modulated by the ganglioside, NeuAcα2-3Galβ1-4Glβ1-ceramide(Cer)(GM1) (Bremer et al, 1986; Weis and Davis, 1990). Thus, it is interesting to note that glycolipids are not only a component of the cell membrane, but can also modulate cell growth.

The extraction of several sulpholipids, a type of glycolipid from marine invertebrates, has been reported (Benson et al, 1959; Benson, 1963; Isono and Nagai, 1965, 1966; Isono et al, 1967; Yoshizaki and Nagai, 1974; Langworthy et al, 1976; Anderson et al, 1978; Kitagawa et al, 1979; Sato et al, 1979; Kikuchi et al, 1982). Gustafson et al (1989) reported that d-sulphonoquinovosyl glycerol from blue-green algae possessed antiviral activity against the human immunodeficiency virus (HIV-1) and cytotoxicity against human lymphocytic cells. This was the first time that sulpholipids were shown to possess antiviral properties. Thus, sulpholipids from marine invertebrates merit further medical study.

In this study, we successfully isolated four sulpholipids from sea urchin intestine, 3'-sulphonoquinovosyl-1', 2'-diacylglyceride (A-4), 3'-sulphononoquinovosyl-1'-monoacylglyceride (2'-lyso A-4, A-5), NeuGcα2-6Glβ1-1Cer (A-6) and HSO3-8NeuGcα2-6Glβ1-1Cer (A-7). The identification of these four sulpholipids had already been reported (Benson et al, 1959; Benson, 1963; Isono and Nagai, 1965, 1966; Isono et al, 1967; Yoshizaki and Nagai, 1974; Langworthy et al, 1976; Anderson et al, 1978; Kitagawa et al, 1979; Sato et al, 1979; Kikuchi et al, 1982; Gustafson et al, 1989; Kubo et al, 1990), although no studies of anti-tumour effect were performed. In the present study, the anti-tumour properties of A-4 and -5 were examined. Sulpholipid A-5 effectively suppresses the growth of solid tumours derived from human lung cancer in vivo, adenocarcinoma cell line A-549, in nude mice.

MATERIALS AND METHODS

Materials

Intestines from the sea urchin, Stronglylocentrotus intermedius, which inhabits the coast of Rishiri Island, Hokkaido, were immersed into acetone overnight and dried (acetone powder).
DEAE-Sephadex A-25 and Sephadex LH-20 were purchased from Pharmacia-LKB (Uppsala, Sweden), Iatrobeads from latron Laboratories (Tokyo, Japan), thin-layer chromatography (TLC) plates (Silica-gel 60) and [3H]dimethylsulphoxide (Me₃SO-d₃) from Merck (Germany). Rhizopus delmer lipase, triacylglycerol acylhydrolase, was obtained from Seikagaku Kogyo Corporation (Tokyo, Japan). Standard glycosphingolipids (GalCer, galactosyleramide; LacCer, lactosylceramide; globotriasylerceramide, Gb₃Cer; and GM₃) were prepared in our laboratory.

Cell line
Cell line W14, which was prepared by transfection with the 6.6-kb EJ-ras oncogene to cell line WFB, derived from WKA rat fibroblast cells, was used (Sato et al, 1987). Human adenocarcinoma A-549 cells from lung cancer were provided by the Japanese Cancer Research Resources Bank. These cells were cultured in Eagle’s minimum essential medium (MEM) (Nissui Co., Tokyo, Japan), which was supplemented with 5% fetal calf serum (FCS) and 2mM L-glutamine (Gibco, Grand Island, NY, USA).

Extraction and purification of glycolipids

The ratio of the solvent mixture is expressed by volume. The glycolipids were extracted three times from 150 g of the acetone powder of the sea urchin intestine with 10 volumes per g of the powder with a chloroform–methanol–water (CMW) ratio of 4:8:3. The crude extracts were combined and evaporated to dryness in vacuo. The dried material was dissolved in CMW (30:60:8), and the solution was passed through a DEAE-Sephadex A-25 column (3.3 × 35 cm, acetate form), which was previously equilibrated with CMW (30:60:8) (Suetake et al, 1993). After washing the column with the equilibration CMW mixture to remove unbound lipids, the bound acidic lipids were eluted with CMW containing 1M ammonium acetate (30:60:8). This acidic fraction was collected, concentrated and passed through a Sephadex LH-20 (1.0 × 35 cm) column to remove ammonium acetate. The total acidic glycolipids were chromatographed on an Iatrobeads column (2.5 × 40 cm) by stepwise elution of increasing polarity with CMW (from 90:10:0.5, 80:20:2, 70:30:3 to 60:40:4, 300 ml each). Aliquots of the fractionated sample were developed with CMW (65:25:4) on a thin-layer chromatography (TLC) plate and visualized with orcin-sulphuric acid. To obtain homogeneous glycolipid, the Iatrobeads chromatography was repeated in the same manner as above, except column size was sequentially decreased. Through the above purification procedure, A-4, -5, -6 and -7 were obtained in amounts of 44.0, 47.4, 49.9 and 44.6 mg respectively, from 150 g of the acetone powder.

Nuclear magnetic resonance

Proton nuclear magnetic resonance (NMR) spectra of the glycolipids (approximately 1 mg) in 0.4 ml of Me₃SO-d₃ containing 2% D₂O were obtained in the Fourier-transform mode on a Varian JNMRAlpha-1 spectrometer at the High Resolution NMR Laboratory, Hokkaido University, as described previously (Suetake et al, 1993). The chemical shift was indicated by distance (p.p.m.) from tetramethylsilane as an internal standard. Two-dimensional chemical shift-correlated spectroscopy (2D-COSY) spectra were obtained as described previously (Suetake et al, 1993) and shown in the absolute value representations as contour plots.

Fast atom bombardment-mass spectrometry

Negative fast atom bombardment-mass spectrometry (FAB-MS) was done on a JEOL JMS-HX100 mass spectrometer equipped with a JMA-DA500 datalizer as described previously (Suetake et al, 1993). The lipid in a matrix of triethanolamine was bombarded by xenon gas with 6 kV (20 mA), and the fragments were accelerated at 5 kV.

Analysis of lipid moieties

The fatty acid components of A-4 and A-5 were separately analysed as methyl esters from the methanolyzates of the purified glycolipids by gas-layer chromatography (GLC) and gas chromatography-mass spectrometry (GS-MS) as reported previously (Suetake et al, 1993).

Decaylation of glycolipid

A-4 (10 mg) with 50 U ml⁻¹ lipase in a solution of 50 mM acetate buffer, pH 5.6, containing 0.1 M calcium chloride was incubated for 72 h at 30 °C. After incubation, the reaction mixture was lyophilized and passed through a Sephadex LH-20 column (1.0 × 30 cm) with CMW (60:30:4.5) to remove salts. The resultant products were isolated by using an Iatrobeads column (1.5 × 40 cm) as described above, giving 0.9, 3.6 and 2.3 mg of unreacted A-4, a lipid (’A-5’) which was identical to natural A-5 by NMR, and sulphonoquinovosylglycerol (SQG) respectively.

MTT assay

MTT, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], assay was performed using W14 and A-549 cell lines according to the method described previously by Takahashi et al (1993). Briefly, these cells (5 × 10⁴ per well) were cultured in
Figure 2 1D- and 2D- NMR spectra of A-4 and -5. A and B show 1D- and 2D- spectra of A-4 respectively.
Antitumour effects of extracts from sea urchin intestine

Figure 2 1D- and 2D- NMR spectra of A-4 and -5. C and D show 1D- and 2D-spectra of A-5. Peaks with asterisks indicate contamination.
Table 1 Fatty acid component (%) of A-4 and A-5

| Fatty acid | A-4  | A-5  |
|------------|------|------|
| 14:0       | 32.5 | 2.4  |
| 14:1       | 1>   | 1>   |
| 16:0       | 57.6 | 86.5 |
| 16:1       | 1>   | 1.0  |
| 18:0       | 2.2  | 5.4  |
| 18:1       | 1>   | 1>   |
| 20:0       | 1>   | ND   |
| 20:1       | ND   | ND   |
| Unknown    | 4.7  | 4.1  |

*Carbon chain length (n) with saturation (0) or mono-unsaturation (1) are abbreviated as n0 or n1. ND, not detected. The component was determined by gas-layer chromatography as the fatty acyl methyl ester.

96-well plates for 24 h and then various amounts of samples suspended in phosphate-buffered saline (PBS) were added to the wells (in the control, PBS was added to wells at a volume of 10% of the medium). Following cultivation for 48 h, 50 μg of MTT was added to the culture medium and incubated for 3 h. Then, 4% hydrochloric acid in isopropanol was added to each well and mixed by pipette to destroy cells. The absorbance of each well was measured using a multiwell scanning photometer (Micro ELISA MR600, Dynatech Laboratories, Alexandria, VA, USA) at a wavelength of 570 nm.

In vivo anti-tumour assay

A-549 cells (5 x 10^4 cells per mouse) were injected subcutaneously into nude mice (BALB/cAJcl-nu). After implantation, the tumour sizes in all of these mice were measured at 3-day intervals. The mice bearing solid tumours that grew to 30–50 mm^3 in tumour volume [tumour volume = length x (width)^2 x 0.5] were used for the anti-tumour assay. They were divided randomly into two groups. A control group was injected with PBS (n=5), and a group was injected with A-5 (n=5) for the period of 32–53 days after implantation. In a separate experiment, eight mice were divided at random into two groups: a control group was injected with PBS (n=4), and a group was injected with A-4 (n=4) for the period of 45–67 days after implantation. These mice were injected subcutaneously eight times at 3-day intervals with A-4 and -5 at a dose of 100 μg per mouse (4 mg kg^-1) in PBS. At the end of the assay, some mice from the control, A-4 and -5 groups were separately examined to determine the histological features of the tumours and major organs, such as lung, heart, spleen, stomach, liver, pancreas, kidney, intestine and brain.

RESULTS

Purification of glycolipid from sea urchin intestine

Whole acidic glycolipid from sea urchin intestine and purified glycolipids are shown on a TLC plate in Figure 1.

NMR analysis of glycolipids

One-dimensional (1D)- and 2D-NMR was done on A-4, -5, -6 and -7. Spectra of A-4 are presented in Figure 2A and B. Typically, the 1D spectrum showed the presence of a diacylglyceride skeleton based on a methylenic proton due to a 2' proton (G-2 in the figure), methylenic protons due to 3' protons (G-3a and -3b) and methylenic protons due to 1' protons (G-1a and -1b) bound on
glyceride carbons. The existence of two fatty acyls was also estimated from four protons resonating at δ 2.25 p.p.m. owing to α-methyleneic protons on acyl groups, the chemical shifts and coupling constants of which were mostly identical to those of glycerophospholipids (Kriat et al., 1993). These protons, as well as other protons, were finally assigned based on the 2D-spectrum, showing that A-4 possesses α-glucose-like ring protons (Q-1 to Q-6). The chemical shifts of the Q-6 methyleneic protons (Q-6a at δ 2.91 p.p.m. and Q-6b at 2.65 p.p.m.) of the α-glucose-like sugar, however, were different from those of α-glucose (H6a at 3.66 and H6b at 3.44; Koerner et al., 1983), suggesting that some functional group other than the hydroxyl residue of glucose was directly bound at the carbon-6 position on the α-glucose-like sugar moiety in A-4. The olefinic protons at δ 5.35 p.p.m. were assigned to the double bond(s) of fatty acid. On the other hand, the 1D-spectrum of A-5 (Figure 2C) also showed the presence of α-glucose-like ring protons (Q-1 to Q-6) but also revealed significant upper-field shifts of G-2 protons, slight shifts of G-3 and G-1 protons, and reduced intensity of α-methyleneic protons on acyl groups compared with A-4. From the shifts and intensities of these protons, the structure of A-5 was determined to be a deacylated derivative of A-4 at C-2' on glyceride. Assignment of the A-5 protons was confirmed by 2D-NMR (Figure 2D). Data for A-6 and -7 are not shown.

**FAB-MS analysis of glycolipids**

To determine the molecular masses of A-4 and -5 together with the functional group attached to the α-glucose-like sugar, negative FAB-MS spectra were measured. A peak at m/z 765 was detected as a molecular ion ([M-H]-) in the spectrum of A-4 (Figure 3A). This indicates that the sugar moiety of A-4 contains a sulphur dioxide component, since the mass is 64 greater than glucose. This was assigned to a sulphono-6-deoxyhexose residue and the acyl moieties were determined by GLC analysis (see below) to be C₁₆ and C₁₄ fatty acids. The peak at m/z 737, as shown in Figure 3A, was assigned to A-4 containing C₁₄ acid and C₁₆ acid moieties. The peak at 555 m/z was assigned to A-4 containing only a C₁₆ acid moiety. Also, for the 765 peak, A-4 was assigned as having two C₁₆ acid components. These fatty acid assignments are not definite, as only C₁₄ to C₂₀ fatty acid components were analysed (see below), although they are most likely. The 555 peak is the main peak in the spectrum of A-5 as shown in Figure 3B. Judging from these FAB-MS peaks as well as the NMR data described above, the chemical structures of A-4 and -5 were identified as 3'-[6-sulphonoquinovosyl] 1', 2'-diacylglyceride and
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Figure 6 In vivo study of anti-tumour effects of A-5 and A-4. To elucidate whether these glycolipids had anti-tumour effects, A-549 cells (5 × 10⁴ cells per mouse) were injected s.c. into nude mice. Mice bearing solid tumours that grew to 30–50 mm³ in tumour volume were used for all experiments. (A) Ten mice were used and divided into two groups; a control group injected with PBS (n=5) and a group injected with A-5 (n=5). (B) Eight mice bearing tumours were divided at random into two groups; a control group (n=4) and an A-4 group (n=4). All mice were injected s.c. eight times at 3-day intervals with A-4 or -5 at a dose of 100 μg per mouse (4 mg kg⁻¹) in PBS. Drug injection periods are indicated. The means (± s.e.) of tumour volumes from each group are shown. (A) The mice injected with A-5 showed significant growth suppression in tumour size at 65 days after tumour implantation (Student's t test, P<0.01). Tumour growth in the mice receiving A-4 was not inhibited (B).

Lipid moieties of glycolipids

The fatty acid components of A-4 and A-5 were analysed separately by GLC and GC–MS, and the data are summarized in Table 1. The major fatty acid component of both A-4 and -5 was saturated C₁₆ acid. C₁₈ acid was also detected from the solvolysate of A-4 as a minor component (32.5%), thus confirming the assignments of the A-4. The fatty acids of A-5 were mainly C₁₆ moieties.

The cytotoxicity of the glycolipids in vitro

The purified glycolipids, A-4, -5, -6 and -7, were added to wells with W14 or A-549 cells at a dose of 100 μg ml⁻¹ and examined three times to determine whether these glycolipids had cytotoxic activity against these tumour cells. As shown in Figure 4A, neither A-4, -6 or -7 influenced the cell growth. Nor did they cause notable morphological changes when observed under a light microscope. On the other hand, most of the cells treated with A-5 shrunk morphologically and were found to be irreversibly destroyed when viewed under a light microscope. Thus, A-5 appears to possess cytotoxicity against cells rather than inhibitory activity. To quantify the cytotoxic activity of A-5, the concentrations for 50% inhibition (IC₅₀) against both cell types were determined. The IC₅₀ values against W14 cells and A-549 cells were found to be 33 μg ml⁻¹ and 35 μg ml⁻¹ respectively (Figure 4B).

Preparation and MTT assay of SQG

To investigate whether the cytotoxicity of A-5 is caused by SQG unit, we prepared SQG by decylation of A-4 with lipase. A-5 was not used to generate SQG, as A-5 possesses cytotoxicity and toxic components of the A-5 could affect the results. As shown in Figure 5, after purification of the digested products by Iatrobeads chromatography, unreacted A-4, a lipid ('A-5') has an identical RF to A-5 and SQG. The structure of the product 'A-5' was confirmed to 1'-acyl derivative by NMR analysis. As expected, the mobility of the SQG was slower than A-5. 1D- and 2D-NMR confirmed the SQG structure, as did FAB-MS (data not shown).

We next performed MTT assays using W14 cells. SQG was added to wells at doses of 50 and 100 μg ml⁻¹. No cytotoxic
activity was observed. These data demonstrate that the hydrophilic derivative of A-4, thus A-5, has no cytotoxicity.

In vivo anti-tumour study of A-4 and -5

Tumour-bearing mice injected subcutaneously with A-5 showed significant suppression (by Student's t-test) of tumour growth about 30 days after injection ($P<0.01$) (Figure 6A) and had no loss of body weight throughout the experimental period (data not shown). Figure 6B presents the results for the A-4-treated group. In the mice with A-4, solid tumour growth was not inhibited. The results for the A-5 group agreed well with the data obtained in vitro. By pathological analysis, the A-5 growth-suppressed tumours were observed to have much larger haemorrhagic necrosis areas compared with controls (Figure 7). The organs, lung, heart, spleen, stomach, liver, pancreas, kidney, intestine and brain, of A-5 treated mice showed a normal histological appearance (data not shown).

DISCUSSION

The intestine absorbs nutrients in a symbiotic relationship with numerousmicrobes. The intestine requires a mechanism regulating the growth of these microbes through various physiologically active substances, because unilateral growth of these microbes can cause the death of the host. This led us to the hypothesis that sea urchin intestine might have some ability to regulate mammalian cell growth, since sea urchins are far removed from both microbes and mammals in terms of evolution. We have isolated and characterized four glycolipids from sea urchin intestine designated A-4, -5, -6 and -7, and confirmed in vivo an anti-tumour effect of one of these lipids, A-5. Several researchers have already reported the isolation and characterization of A-4 and -5 from a bacillus, a diatom, a blue-green algae, a marine sponge and sea urchin gametes (Benson et al, 1959; Benson, 1963; Isono and Nagai, 1965, 1966; Isono et al, 1967; Yoshizaki and Nagai, 1974; Langworthy et al, 1976; Anderson et al, 1978; Kitagawa et al, 1979; Sato et al, 1979; Kikuchi et al, 1982; Gustafson et al, 1989), and of A-6 and -7 from sea urchin gametes (Kubo et al, 1990). It remains unknown whether A-4 and -5 originate from sea urchin intestine or from ingested organisms, since these glycolipids are also extracted from diatoms and algae, which sea urchins feed on.

There have been several reports concerning the physiological effects of lysosphingolipids. For example, lysosphingolipids regulate protein kinase C activity (Hamun and Bell, 1986; Oishi et al, 1988; Merrill and Stevens, 1989), inhibit growth of neuroblastoma cells and influence neurite outgrowth of these cells (Sugiyama et al, 1990; Uehara et al, 1991). Gustafson et al (1989) reported that sulpholipids extracted from blue-green algae, one of which is thought to be almost identical to A-4 in this study, possess antiviral activity against HIV-1 and cytotoxicity against a human lymphocytic cell line. The fact that the 'A-4' from blue-green algae showed activity against human lymphocytes, while sea urchin A-4 showed no activity against the tumour cells used in this study can be attributed to two possibilities: (1) cell type; or (2) acyl groups of 'A-4' and A-4 are slightly different in regard to chain length and/or saturation. The common SQG, sulphonquinovosylglyce-rol, backbone of A-4, A-5 and 'A-4' was generated from A-4 following lipase treatment and found to have no cytotoxic properties. Therefore, the difference(s) in fatty acid composition between A-4 and 'A-4' is (are) responsible for the cytotoxic effect.

The cytotoxicity in vitro of lysolecithin is via haemolytic effect, like a surfactant, that accelerates the permeability of the lipid bilayer responsible for easy incorporation of this molecule into the membrane (Matamoto, 1961; Robinson, 1961; Gottfried and Rapport, 1963). Taketomi et al (1976) reported that lysosphingolipid had strong haemolytic activity compared with the corresponding sphingolipid. When the structural properties of A-4 and -5 are compared, A-5, which is a mono-acetylated structure of A-4, may be more easily incorporated than A-4 into cell membranes, similar to the observations by Taketomi et al (1976). Thus, the common structural characteristic of A-5 and lysosphingolipid is the presence of a single long chain hydrocarbon – the lyso form is cytotoxic in this study and Taketomi's study.

In vivo, A-5 significantly suppressed the growth of solid tumours of human adenocarcinoma derived from lung cancer, but A-4 did not. These results are similar to those obtained in vitro. The tumours of mice administered A-5 were observed to have much larger haemorrhagic necrosis areas, but tumour-infiltrating lymphocytes were not markedly increased compared with the control. The subcutaneous administration sites did not show any tissue disorder. Therefore, the striking suppression seemed to be caused by directly inducing haemorrhagic necrosis. It has been reported that DT-5461, a lipid A analogue, has an indirect anti-tumour effect by inducing endogenous tumour necrosis factor (Sato et al, 1995). In addition to the suppressive effect on the cytotoxic activity in vitro, however, A-5 may have a direct in vivo effect via haemorrhagic necrosis by which tumour growth is inhibited. Further study is required to determine whether A-5 induces tumour necrosis factor.

This is the first time a lysoglycoglycerolipid has been shown to possess anti-tumour activity in vivo. Therefore, this class of compounds should be more thoroughly investigated for drug use, specifically cancer chemotherapy. We are currently studying the pharmacological effects of A-5 in vivo as its direct effect on tumours could prove to be extremely useful in a variety of contexts.

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