Effects of Phosphorylation of Immunomodulatory Agent FTY720 (Fingolimod) on Antiproliferative Activity against Breast and Colon Cancer Cells

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FTY720 (fingolimod), a novel immunosuppressant, was found to become biologically activated by phosphorylation into FTY720-1-phosphate (FTY720-P), which is a high-affinity agonist for sphingosine-1-phosphate (sphingosine-1-P)-receptors. FTY720 has also been reported to have a strong antitumor activity. The association between the phosphorylation of FTY720 and the growth inhibition of FTY720 against cancer cells are still not completely understood. In this study, we investigated the effects of FTY720, sphingosine, and their related compounds on the proliferation of human breast cancer cell lines (MCF-7, MDA-MB-231 and Sk-Br-3) and human colon cancer cell lines (HCT-116 and SW620). Non-phosphorylated FTY720, sphingosine and an FTY720 derivative, ISP-I-55, showed significant growth inhibition against these cells, with IC_{50} values of 5—20 μM at 48 h post-drug treatment. We confirmed that FTY720 induces the activation of a major mitogen-activated protein kinase, JNK, without the activation of p38 and down-regulation of phospho-ERK in MCF-7 breast cancer cells. In contrast, the phosphorylated derivatives, FTY720-P and sphingosine-1-P, as well as a phosphinane FTY720 derivative, cFTY720-P, did not inhibit the growth of the cells in the concentration range of 5—50 μM, whereas FTY720-P and sphingosine-1-P slightly induced the growth of MCF-7 cells. Combining FTY720 with dimethylsphingosine, a sphingosine kinase inhibitor, augmented the inhibitory effect of FTY720. These results indicate that the antiproliferative activity of FTY720 does not result from its phosphorylation, either endogenous or exogenous.

Key words FTY720; fingolimod; antiproliferation; breast cancer; colon cancer; MCF-7

A group of our co-workers (Fujita *et al.*) discovered a potent immunosuppressant, ISP-I[1] (identical with the antifungal antibiotics myriocin[2,3] and thermozymocidin[4,5]), in the entomopathogenic fungus *Isaria sinclairii*, and were led to FTY720 (fingolimod), which is in the late stages of a clinical trial for multiple sclerosis treatment.[6] FTY720 inhibits the sphingosine-1-phosphate (sphingosine-1-P) receptor–dependent egress of lymphocytes from lymph nodes into efferent lymphatics and blood. In this process, FTY720 was found to be monophosphorylated by sphingosine kinase type 2 into FTY720-1-phosphate (FTY720-P), which down-modulates the sphingosine-1-P receptor on lymphocytes.[7—9]

FTY720 has also been reported to have selective cytotoxicity against human cancer cell lines including multiple myeloma,[10] prostate cancer,[11] bladder cancer,[12] hepatoma,[13] breast cancer[14] and leukemia cells.[15] The cells incubated with FTY720 demonstrated features characteristic of apoptosis. However, the association between the phosphorylation of FTY720 and its cytotoxicity against cancer cells is not clear. Thus, we tested the effect of the phosphorylation of FTY720 and its related compounds (Fig. 1) on the proliferation of human breast cancer cell lines (MCF-7, MDA-MB-231 and Sk-Br-3) and human colon cancer cell lines (HCT-116 and SW620). We also examined the influence of endogenous sphingosine kinases on the cytotoxicity of FTY720 using *N*,*N*-dimetylsphingosine (DMS),[16] an inhibitor of sphingosine kinase. Furthermore, we examined the influences of FTY720 and sphingosine on the activation of stress activated mitogen-activated protein kinases (MAPKs), c-Jun NH_2-terminal kinase (JNK) and p38, as well as a survival MAPK, extracellular signal-regulated kinase (ERK), in MCF-7 cells.

MATERIALS AND METHODS

General Procedure IR spectra were recorded on Shimadzu FTIR-8400 infrared spectrophotometer. FAB-MS spectra were measured on a JEOL JMS-HX 100 instrument. Electrospray ionization mass spectra (ESI-MS) were measured on an API-3000 mass spectrometer (Applied Biosystems) fitted with a Turboionspray™ interface. ^{1}H-NMR spectra were recorded on JEOL EX-400 (400 MHz) instruments using tetramethylsilane as an internal standard. Analytical TLC and preparative TLC were performed using Silica gel 60 F254 (Merck, 0.25 and 0.5 mm, respectively) glass plates. Column chromatography was performed using Silica Gel 60 (70—230 mesh ASTM). All extracted solvents were dried over Na_2SO_4, followed by evaporation *in vacuo*.

Materials and Chemicals Sphingosine and DMS were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and sphingosine-1-P was obtained from the Kanto Chemical Co., Inc. (Tokyo, Japan). ISP-I-55 was prepared previously by one of our co-workers (T. Fujita).[17] FTY720

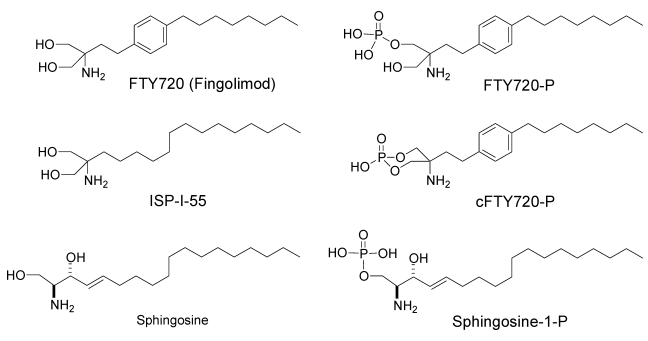

Fig. 1. FTY720 (Fingolimod) and Related Compounds Used in This Study

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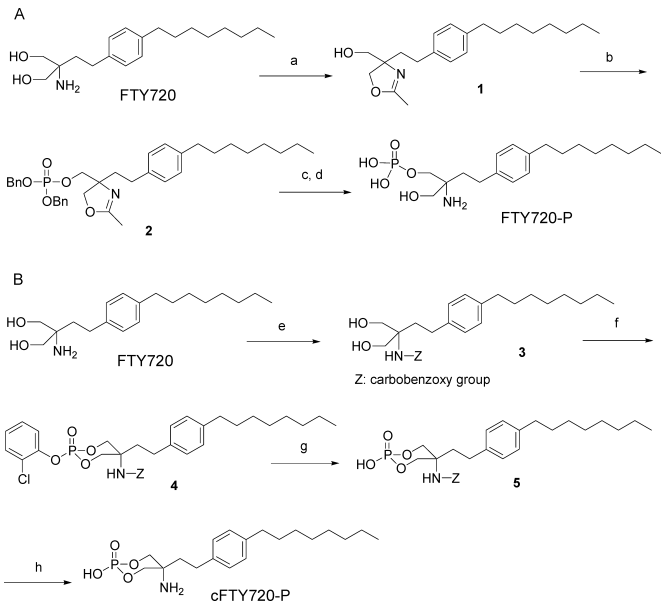

Fig. 2. Synthetic Scheme for FTY720-P (A) and cFTY720-P (B)

Conditions: (a) triethyl orthoacetate, DIEA; (b) $(BnO)_2PN(iso\text{-}Pro)_2$, 1*H*-tetrazole and then I_2; (c) H_2, 10% Pd/C; (d) conc HCl; (e) Z-Cl; (f) 2-chrolophenylphosphorodichroridate; (g) 2-nitrobenzoaldxime, 1,1,3,3-tetramethylguanidine; (h) H_2, 10% Pd/C.

was synthesized according to a method previously detailed.[18,19] FTY720-P[20] and cFTY720-P[21] were synthesized starting from FTY720, as shown in Fig. 2. The spectroscopic data were identical to that reported previously. Precise synthetic methods for the preparation of FTY720-P, cFTY720-P and the intermediates are described hereafter.

{2-Methyl-4-[2-(4-octylphenyl)ethyl]-4,5-dihydro-1,3-oxazol-4-yl}methanol (1)[20] A solution of FTY720 (1.30 g, 4.2 mmol), *N*,*N*′-diisopropylethylamine (1.09 ml, 6.3 mmol) and triethyl orthoacetate (0.93 ml, 5.1 mmol) in DMF (10 ml) was stirred at 120 °C for 2 h. The solution was extracted with EtOAc, washed with sat. NaCl and dried. Concentration and subsequent SiO_2 column chromatography (chloroform/methanol=9/1) gave **1** (1.20 g, 86%) as a colorless oil. ^{1}H-NMR ($CDCl_3$) δ: 7.08 (4 H, s), 4.22 (1H, d, J=8.4 Hz), 4.06 (1H, d, J=8.4 Hz), 3.68 (1H, d, J=11.0 Hz), 3.44 (1H, dd, J=11.0, 7.8 Hz), 2.61—2.49 (4H, m), 2.10—2.04 (1H, m), 2.02 (3H, s), 1.88 (1H, ddd, J=13.6, 11.4, 5.8 Hz), 1.75 (1H, ddd, J=13.6, 11.0, 5.8 Hz), 1.58 (2H, quint, J=7.5 Hz), 1.34—1.23 (10H, m), 0.87 (3H, t, J=6.8 Hz). ESI-MS *m*/*z*: 332 ($M+H^+$).

Dibenzyl {2-Methyl-4-[2-(4-octylphenyl)ethyl]-4,5-dihydro-1,3-oxazol-4-yl}methyl Phosphate (2) To a solution of **1** (57.7 mg, 0.174 mmol) in CH_2Cl_2 (1.1 ml) was added 0.45 M 1*H*-tetrazole in CH_3CN (0.77 ml) and *N*,*N*′-diisopropyldibenzylphospholamidite (74 μl, 0.23 mmol). After being stirred for 1 h the reaction mixture was added to a 10% solution of I_2 in THF/pyridine/H_2O=66/1/33. 5% Na_2SO_3 aq. was added and extract with $CHCl_3$, wash with brine and dried. Concentration and subsequent separation with preparative SiO_2 TLC (EtOAc/methanol=9/1) gave **2** (44.7 mg, 44%) as a colorless oil. ^{1}H-NMR ($CDCl_3$) δ: 0.856 (3H, t, J=7.19 Hz), 1.25—1.50 (10H, m), 1.862 (2H, d, J=9.59 Hz), 1.91—1.99 (2H, m), 2.02 (3H, s), 2.45—2.58 (4H, m), 3.65—4.25 (2H, m), 4.77—4.86 (2H, m), 6.86—7.23 (14H, m). ESI-MS *m*/*z*: 592.8 ($M+H^+$), HR-FAB-MS *m*/*z*: 592.3210 (Calcd for $C_{35}H_{47}NO_5P$: 592.3192).

FTY720-P[20] A solution of **2** (16.5 mg, 0.028 mmol) in methanol/acetic acid=1/1 (4.0 ml) was stirred over 10% palladium-on-charcoal (16 mg) under H_2 atmosphere for 7 h at rt. After removal of the catalyst by filtration, the filtrate was concentrated. The residue was dissolved in conc HCl/ethanol=1/10 (0.18 ml) and the solution was stirred at 50 °C for 6 h. 0.47 ml of water was added and stirred at room temperature (rt) for over night to form a precipitate of HCl·FTY-720-P. The precipitate was collected by filtration, washed with EtOAc. The compound (8.3 mg, 76%) was used for the biological tests without further purification. ^{1}H-NMR (CD_3OD) δ: 0.871 (3H, t, J=3.20 Hz), 1.22—1.29 (10H, m), 1.58 (2H, s), 1.92—1.98 (2H, m), 2.54—2.65 (2H, m), 3.47—3.74 (2H, m), 4.08—4.30 (2H, m), 7.00—7.18 (4H, m). ESI-MS *m*/*z*: 388.5 ($M+H^+$).

Benzyl 2-(2-Chlorophenoxy)-5-[2-(4-octylphenyl)ethyl]-2-oxido-1,3,2-dioxaphosphinan-5-ylcarbamate (4) To a solution of **3**[20] (200 mg, 0.45 mmol) in dioxane (2.72 ml) was added 2-chlorophenylphosphordichloridate (0.117 ml, 0.68 mmol) and pyridine (0.110 ml, 1.36 mmol). Being stirred at rt for 22 h, the solution was concentrated and dissolved in $CHCl_3$ (15 ml). The solution was washed with water and dried. Concentration and subsequent separation with preparative SiO_2 TLC ($CHCl_3$/methanol=9/1) gave **4** (171.6 mg, 62%) as a colorless oil. ^{1}H-NMR ($CDCl_3$) δ: 0.875 (3H, t, J=6.5 Hz), 1.14—1.41 (10H, m), 1.57 (2H, t, J=7.3 Hz), 2.00 (2H, t, J=8.1 Hz), 2.22 (2H, t, J=8.1 Hz), 2.54 (4H, t, J=7.8 Hz), 4.34 (2H, dd, J=11.1, 17.3 Hz), 4.42 (2H, d, J=11.3 Hz), 4.59 (2H, dd, J=11.3, 24.0 Hz), 4.79 (2H, dd, J=8.9, 11.1 Hz), 5.04 (2H, s), 5.06 (1H, s), 5.10 (2H, s), 6.94—7.49 (13H, m). ^{13}C-NMR ($CDCl_3$) δ: 14.2, 22.7, 28.7, 29.3, 29.5, 31.6, 31.8, 31.9, 32.7, 35.5, 53.0 and 53.1 (conformer), 53.6 and 53.7 (conformer), 66.9 and 67.0 (conformer), 72.4 (J_{C-P}=6.7 Hz) and 73.8 (J_{C-P}=7.8 Hz, conformer), 120.9, 121.2, 126.0, 127.9, 128.0, 128.1, 128.2, 128.4, 128.5, 130.5, 135.7, 135.9, 137.2, 137.3, 140.9, 146.0 and 146.1 (conformer), 145.7 and 145.8 (conformer), 154.4, 154.7. IR (KBr) cm^{-1}: 1236, 1309, 1497, 1724, 2854, 2926, 3292, 3568, 3587. FAB-MS *m*/*z*: 614 ($M+H^+$), 277, 255, 209, 185, 143, 117, 91. HR-FAB-MS *m*/*z*: 614.2431 (Calcd for $C_{33}H_{42}ClNO_6P$: 614.2438).

Benzyl 2-Hydroxy-5-[2-(4-octylphenyl)ethyl]-2-oxido-1,3,2-dioxaphosphinan-5-ylcarbamate (5) A solution of 2-nitrobenzaldoxime (39.7 mg, 0.24 mmol), 1,1,3,3-tetramethylguanidine (59.7 μl, 0.48 mmol) in dioxane (0.5 ml) was added to a solution of **4** (69.6 mg, 0.12 mmol) in dioxane (0.6 ml) and stirred for 44 h at rt. After removal of the solvent by evaporation, the residue was dissolved in methanol and treated with SEPHADEX IR-120 ion exchange resins. Concentration and subsequent separation with preparative SiO_2 TLC ($CHCl_3$/methanol=7/3) gave **5** (43 mg, 75%) as a colorless oil. ^{1}H-NMR (CD_3OD) δ: 0.89 (3H, t, J=6.8 Hz), 1.19—1.40 (10H, m), 1.57 (2H, t, J=6.8 Hz), 1.93 (2H, t, J=6.8 Hz), 2.53 (4H, t, J=6.8 Hz), 4.03—4.41 (4H, m), 5.07 (2H, s), 6.91—7.07 (4H, m), 7.22—7.43 (5H, m). ^{13}C-NMR (CD_3OD) δ: 14.5, 23.7, 29.9, 30.3, 30.4, 30.6, 32.8, 33.0, 33.6, 36.5, 55.0, 67.0, 72.0, 128.7, 128.8, 129.1, 129.2, 129.3, 140.1, 141.3, 157.1. IR (KBr) cm^{-1}: 122.8, 1506.3, 1712.2, 2358.8, 2854.5, 2927.7, 3018.4, 3647.1, 3674.1. FAB-MS *m*/*z*: 504 ($M+H^+$), 440, 414, 392, 370, 255, 239,

223, 207, 155, 115, 91. HR-FAB-MS *m/z*: 504.2516 (Calcd for $C_{27}H_{39}NO_6P$: 504.2515).

5-Amino-5-[2-(4-octylphenyl)ethyl]-1,3,2-dioxaphosphinan-2-ol 2-oxide (cFTY720-P)[21] A solution of **5** (45.8 mg, 0.091 mmol) in ethanol (5 ml) was stirred under H_2 atmosphere on 10% palladium-on-charcoal (4.5 mg) for 21 h at rt. After removal of the catalyst by filtration, the filtrate was concentrated to afford cFTY720-P (28.9 mg, 86%) as a colorless oil. ^{1}H-NMR (CD_3OD) δ: 0.798 (3H, t, J=5.95 Hz), 1.16—1.69 (26H, m), 3.72 (2H, dd, J=10.8, 18.1 Hz), 4.04 (2H, dd, J=6.2, 10.8 Hz). ^{13}C-NMR (CD_3OD) δ: 14.5, 23.6, 23.8, 30.5, 30.6, 30.7, 30.8, 31.5, 33.1, 35.1, 40.1, 51.6, 75.4 (J_{C-P}=5.6 Hz). IR (KBr) cm^{-1}: 1218, 1503.3, 2358.8, 2854.5, 2927.7, 3018.4, 3637.1, 3574.1. FAB-MS *m/z*: 370 ($M+H^+$), 286, 255, 203, 185, 155, 143, 115, 93, 91.

Cell Lines and Culture Conditions All of the cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, Virginia). The human breast carcinoma cell lines MCF-7, MDA-MB-231 and Sk-Br-3 were maintained in an RPMI-1640 medium (Sigma-Aldrich Co., St. Louis, MO, U.S.A.) with 10% fetal bovine serum (Gibco BRL, Grand Island, NY, U.S.A.) and 50 U/ml ampicillin at 37 °C in a humidified atmosphere containing 5% CO_2. The human colon carcinoma cell lines HCT-116 and SW620 were maintained in the same conditions except that McCoy's 5A medium (Invitrogen, Carlsbad, CA, U.S.A.) was exploited.

***In Vitro* Proliferation Assay** Cells were seeded onto 96-well plates at the densities of 3000 cells/well. After 24 h of pre-cultivation, appropriate concentrations of the samples were added and the cells were cultured for 2 d. The relative growth ratio was determined by WST-1 assay using a cell counting kit (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. The IC_{50} value was determined from the dose response curve. At least three independent experiments were performed. The proliferation assay that tested the effects of DMS was performed using identical methods. Compounds with or without DMS were added to the wells and the plates were incubated for 78 h at 37 °C in a humidified atmosphere containing 5% CO_2 before the WST-1 assay was conducted.

Western Blot MCF-7 cells on a 60-mm plate were cultivated for 24 h in a serum-free medium prior to the addition of the test compounds. The cells were incubated with FTY720 (10 μM) or sphingosine (20 μM) in a PRMI-1640 medium. After 0.5, 2, 4, 8, 12, or 24 h the medium was removed and the cells were washed twice with PBS. The cells were lysed and protein extraction was performed. The components of the lysis buffer were as follows: 1% Nonidet P-40 (Roche Diagnostics GmbH, Penzberg, Germany), 0.1% SDS, 0.05% sodium deoxycholate, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 100 mM NaCl, 20 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1 mM NaF, 1.2 mg aprotinin, 0.2 mg leupeptin, 0.02 mg pepstatin, and 1 mM PMSF in 10 mM Tris–HCl (pH 8.0). The samples were separated by SDS-PAGE (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan) electrophoretically transferred to a polyvinylidene difluoride membrane (Pall Co., East Hills, NY, U.S.A.) and labeled with rabbit polyclonal anti-ERK, anti-phospho-ERK, anti-JNK, anti-phospho-JNK, anti-p38, anti-phospho-p38 and anti-actin. They were visualized with peroxidase-conjugated second antibodies using a standard ECL kit (Amersham, Inc., Arlington Heights, IL, U.S.A.).

Table 1. Inhibitory Effects of FTY720, Sphingosine and ISP-I-55 on Cancer Cell Proliferation, Determined by WST-1 Assay

Cell line	IC_{50} (μM)		
	FTY720	Sphingosine	ISP-I-55
MCF-7	6.8±0.6	12.6±1.4	8.5±1.5
MDA-MB-231	6.7±0.5	14.7±0.8	12.3±0.7
Sk-Br-3	5.8±1.3	18.2±0.7	17.1±0.7
HCT-116	5.7±1.2	17.0±0.9	16.4±1.4
SW620	6.7±0.6	6.8±1.2	6.6±1.2

IC_{50} values, the concentrations of tested compounds causing 50% growth inhibition in the WST-1 assay 48 h after treatment, were determined by linear interpolation between the values immediately above and below the 50% inhibition. Values are means±S.D. from at least three independent experiments.

RESULTS

Inhibitory Effect of FTY720, Sphingosine and ISP-I-55 on Cancer Cell Proliferation A WST-1 assay was performed to determine the IC_{50} values of FTY720 and sphingosine against the proliferation of human breast cancer cell lines, MCF-7, MDA-MB-231, and Sk-Br-3 as well as human colorectal cancer cell lines, HCT-116 and SW620. The results demonstrated that treatment of the compounds caused cell death in a dose-dependent manner. As shown in Table 1, FTY720 exhibited comparatively low IC_{50} values within the concentration range of 5—7 μM for all of the cells tested in this study. Judging from the values, the effects of sphingosine were two to three times lower than those of FTY720, except for the comparative effects on SW620 cells. We also tested an analog of FTY720, ISP-I-55, which lacks an aromatic ring in the aliphatic chain region. ISP-I-55 induced dose-dependent growth inhibition against breast cancer cell lines and colon cancer cell lines with IC_{50} values comparable to those of sphingosine.

Effect of Sphingosine Kinase Inhibitor DMS on the Antiproliferative Activity of FTY720 It is known that FTY720 needs to be mono-phosphorylated by sphingosine kinase in the cytosol in order to become biologically active on sphingosine-1-P-receptors, S1P-R_1 and S1P-R_3.[9] We used a potent inhibitor of sphingosine kinase, DMS,[16] to clarify the effects of the phosphorylation of FTY720 on the proliferation of a breast cancer cell line, MCF-7, on which S1P-R_1 and S1P-R_3 are highly expressed.[22] FTY720 in various concentrations with or without 10 μM DMS was treated with MCF-7 cells for 78 h and the cell viabilities were measured (Fig. 3). The graph shows that the antiproliferative activity of FTY720 was enhanced rather than inhibited by co-treatment with DMS, suggesting that the endogenous phosphorylation of FTY720 is not required for its cytotoxicity. The increased activity may result from the increased amount of intact FTY720 resulting from the inhibition of its phosphorylation.

Effect of Phosphorylation of FTY720 on Cancer Cell Proliferation In order to investigate the effects of exogenous phosphorylated FTY720, we synthesized FTY720-P and a phosphatase-tolerant phosphinane FTY720 derivative, cFTY720-P. The IC_{50} values of these agents against breast cancer cell lines (MCF-7, MDA-MB-231, Sk-Br-3) and colon cancer cell lines (HCT-116, SW620) were evaluated

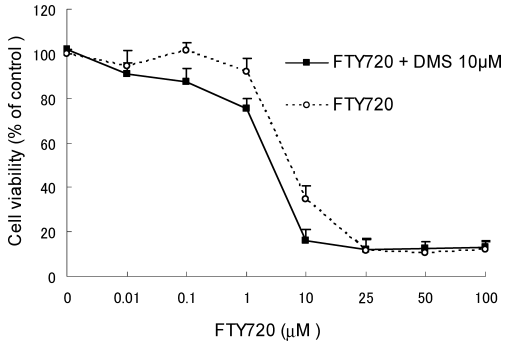

Fig. 3. Effect of Sphingosine Kinase Inhibitor DMS on Antiproliferative Activity of FTY720 against Breast Cancer Cell Line MCF-7

Compounds with and without DMS (10 μM) were added to the wells and the plates were incubated for 78 h (37 °C, 5% CO_2) before a WST-1 assay was conducted. Bars represent the S.D. of triplicate assays.

Table 2. Effect of Phosphorylated FTY720 Derivatives, FTY720-P and cFTY720-P on Cancer Cell Proliferation

Cell line	IC_{50} (μM)	
	FTY720-P	cFTY720-P
MCF-7	79.1±2.0	>100
MDA-MB-231	59.9±9.2	>100
Sk-Br-3	72.9±7.7	>100
HCT-116	>100	>100
SW620	40.0±12.1	>100

IC_{50} values were determined by linear interpolation between the values immediately above and below the 50% inhibition. The relative growth ratio of the cells was determined by WST-1 assay 48 h after the treatment of the test compounds. Values are means±S.D. from at least three independent experiments.

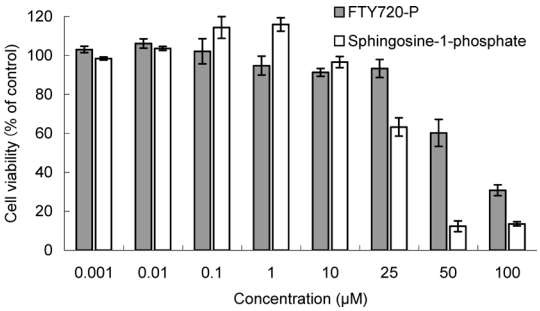

Fig. 4. Concentration Dependence of Cell Viabilities of MCF-7 Cells Treated with FTY720-P and Sphingosine-1-P

The relative growth ratio of the cells was determined by a WST-1 assay conducted 48 h after the treatment of the test compounds. Values are means±S.D. from at least three independent experiments.

using a WST-1 assay (Table 2). Both compounds showed considerably high IC_{50} values in comparison to FTY720. The low concentration range of FTY720-P induced cell growth slightly in MCF-7. This tendency was stronger for sphingosine-1-P, although this compound was more cytotoxic in a higher concentration range (Fig. 4).

The Effect of FTY720 and Sphingosine on the Activation of Major MAPKs in MCF-7 Cells We evaluated the effect of FTY720 (10 μM) and sphingosine (20 μM) on the phosphorylation of major MAPKs, survival MAPK/extracellular signal-regulated kinase, ERK1/ERK2, the stress-activated MAPK c-Jun NH_2-terminal kinase, JNK1/JNK2 and the death kinase p38 in a human breast cancer cell line,

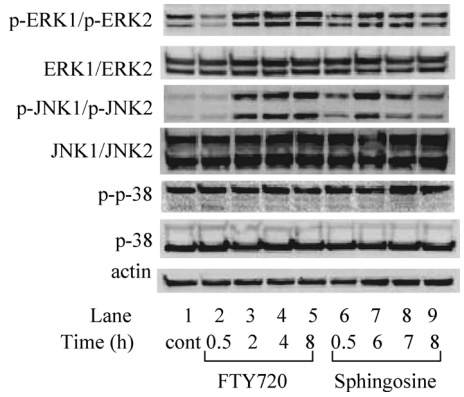

Fig. 5. Effects of FTY720 and Sphingosine on Phosphorylation of ERK1/ERK2, JNK1/JNK2 and p38 in MCF-7 Cells

Cultured cells were incubated with 10 μM FTY720 for 0.5 h (lane 2), 2 h (lane 3), 4 h (lane 4) and 8 h (lane 5) and with 20 μM sphingosine for 0.5 h (lane 6), 2 h (lane 7), 4 h (lane 8) and 8 h (lane 9). The cell extracts were examined by Western blotting using the respective antibodies as described in Materials and Methods. An actin antibody served as the loading control.

MCF-7. Treatment of MCF-7 cells with FTY720 for 0.5 h caused a strong down-regulation of phosphorylated ERK1/ERK2 (p-ERK1/p-ERK2), whereas long term incubation caused a recovery of p-ERK1/p-ERK2 expression level. The phosphorylation of ERK was also inhibited by treatment of the cells with sphingosine for 0.5 h. On the other hand, the phosphorylation of JNK1/JNK2 was induced 2—8 h after the addition of FTY720 or sphingosine. Both FTY720 and sphingosine led to the activation of JNK1/JNK2, but not of p38 (Fig. 5).

DISCUSSION

We evaluated the antiproliferative effects of FTY720, sphingosine and their analogues on human breast cancer cell lines MCF-7, MDA-MB-231, and Sk-Br-3 as well as human colorectal cancer cell lines HCT-116 and SW620. FTY720, sphingosine and ISP-I-55 inhibited the growth of these cells. FTY720 showed the highest cytotoxicity among the tested compounds. ISP-I-55 has a structure lacking a benzene ring found in FTY720. Judging from the mouse allogeneic mixed lymphocyte reaction,[17] the immunosuppressive effect of ISP-I-55 is less active than that of FTY720. A similar structure–activity relationship has been shown in the cytotoxicity between these two compounds. Western blot analysis of the cytosolic fraction of MCF-7 cells demonstrated that 10 μM FTY720 or 20 μM sphingosine temporally deactivated the phosphorylation of a survival MAPK, ERK, and activated the phosphorylation of death kinase, JNK without modulation of p-p38. The recovery of p-ERK expression after the temporal down-regulation would be associated with the regulation of apoptosis or survival signaling in the cells.[23,24] The results suggested that the antiproliferation of FTY720 and sphingosine proceeded in a similar manner including the induction of apoptosis. Although FTY720-P and cFTY720-P are active mediators in the immunomodulation and endothelial cell functions, these compounds affected little to the cell growth. Observation of the low cytotoxicity of FTY720-P may be caused by endogenous dephosphorylation, since phosphatase-tolerant cFTY720-P has much less of an effect. Furthermore, the pretreatment of DMS did not inhibit the ef-

fects of FTY720, but it increased the effect of FTY720. This may be caused by the increased amount of intact FTY720 resulting from the inhibition of the phosphorylation with an effect of DMS. The results indicate that neither the endogenous nor the exogenous phosphorylation of FTY720 is involved in the cytotoxicity of the compound.

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REFERENCES

1) Kiuchi M., Adachi K., Kohara T., Minoguchi M., Hanano T., Aoki Y., Mishina T., Arita M., Nakao N., Ohtsuki M., Hoshino Y., Teshima K., Chiba K., Sasaki S., Fujita T., *J. Med. Chem.*, **43**, 2946—2961 (2000).
2) Kluepfel D., Bagli J., Baker H., Charest M.-P., Kudelski A., Sehgal S. N., Vezina C. *J. Antibiot.*, **25**, 109—115 (1972).
3) Bagli J. F., Kluepfel D., St-Jacques M., *J. Org. Chem.*, **38**, 1253—1260 (1973).
4) Craveri R., Manachini P. L., Aragozzini F., *Experientia*, **28**, 867—868 (1972).
5) Aragozzini F., Manachini P. L., Craveri R., Rindone B., Scolastico C., *Tetrahedron*, **28**, 5493—5498 (1972).
6) Kappos L., Antel J., Comi G., Montalban X., O'Connor P., Polman C. H., Haas T., Korn A. A., Karlsson G., Radue E. W., *N. Engl. J. Med.*, **355**, 1124—1140 (2006).
7) Brinkmann V., Davis M. D., Heise C. E., Albert R., Cottens S., Hof R., Bruns C., Prieschl E., Baumruker T., Hiestand P., Foster C. A., Zollinger M., Lynch K. R., *J. Biol. Chem.*, **277**, 21453—21457 (2002).
8) Mandala S., Hajdu R., Bergstrom J., Quackenbush E., Xie J., Milligam J., Thornton R., Shei G.-J., Card D., Keohane C., Rosenbach M., Hale J., Lynch C. L., Rupprecht K., Parsons W., Rosen H., *Science*, **296**, 346—349 (2002).
9) Matloubian M., Lo C. G., Cinamon G., Lesneski M. J., Xu Y., Brinkmann V., Allende M. L., Proia R. L., Cyster J. G., *Nature* (London), **427**, 355—360 (2004).
10) Yasui H., Hideshima T., Raje N., Roccaro A. M., Shiraishi N., Kumar S., Hamasaki M., Ishitsuka K., Tai Y., Podar K., Catley L., Mitsiades C. S., Richardson P. G., Albert R., Brinkmann V., Chauhan D., Anderson K. C., *Cancer Res.*, **65**, 7478—7484 (2005).
11) Permpongkosol S., Wang J., Takahara S., Matsumiya K., Nonomura N., Nishimura K., Tsujimura A., Kongkanand A., Okuyama A., *Int. J. Cancer*, **98**, 167—172 (2002).
12) Azuma H., Takahara S., Horie S., Muto S., Otsuki Y., Katsuoka Y., *J. Urol.*, **169**, 2372—2377 (2003).
13) Lee T. K., Man K., Ho J. W., Sun C. K., Ng K. T., Wang X., Wong Y., Ng I. O., Xu R., Fan S. T., *Carcinogenesis*, **25**, 2397—2405 (2004).
14) Azuma H., Horie S., Muto S., Otsuki Y., Matsumoto K., Morimoto J., Gotoh R., Okuyama A., Suzuki S., Katsuoka Y., Takahara S., *Anticancer Res.*, **23**, 3183—3193 (2003).
15) Matsuoka Y., Nagahara Y., Ikekita M., Shinomiya T., *Br. J. Pharmacol.*, **138**, 1303—1312 (2003).
16) Edsall L. C., Van Brocklyn J. R., Cuvillier O., Kleuser B., Spiegel S., *Biochemistry*, **37**, 12892—12898 (1998).
17) Fujita T., Hirose R., Yoneta M., Sasaki S., Inoue K., Kiuchi M., Hirase S., Chiba K., Sakamoto H., Arita M., *J. Med. Chem.*, **39**, 4451—4459 (1996).
18) Mishina T., Ohara T., Adachi K., Jpn. Kokai Tokkyo Koho, JP11310556 (1999).
19) Adachi K., Kohara T., Nakao N., Akita M., Chiba K., Mishina T., Sasaki S., Fujita T., *Bioorg. Med. Chem. Lett.*, **5**, 853—856 (1995).
20) Kiuchi M., Adachi K., Tomatsu A., Chino M., Takeda S., Tanaka Y., Maeda Y., Sato N., Mitsutomi N., Sugahara K., Chiba K., *Bioorg. Med. Chem.*, **13**, 425—432 (2005).
21) Chino M., Adachi K., Tanaka Y., Sugahara K., Matsuyuki H., Tomatsu A., Kiuchi M., WO 2005014603 (2005) [*PCT Int. Appl.*].
22) Wang F., Van Brocklyn J. R., Edsall L., Nava V. E., Spiegel S., *Cancer Res.*, **59**, 6185—6191 (1999).
23) Boldt S., Weidle U. H., Kolch W., *Carcinogenesis*, **23**, 1831—1838 (2002).
24) Park H. J., Kim B. C., Kim S. J., Choi K. S., *Hepatology*, **35**, 1360—1371 (2002).