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Communication

Sphingosine 1-phosphate (S1P) induces shape change in rat C6 glioma cells through the $S1P_2$ receptor: development of an agonist for S1P receptors

Kyeok Kim, Yu-Lee Kim, Santosh J. Sacket, Hyo-Lim Kim, Mijin Han, Deok Seong Park, Baeck Kyoung Lee, Won Koo Lee, Hyun-Joon Ha and Dong-Soon Im

Abstract

Treatment with isoprenaline led to a change in the cell morphology of rat C6 glioma cells. This morphological change was reverted by the addition of sphingosine 1-phosphate (S1P). Using this morphological change as a response marker we determined that DS-SG-44 ((2*S*,3*R*)-2-amino-3-hydroxy-4-(4-octylphenyl)butyl phosphoric acid) was an agonist of S1P receptors. The DS-SG-44-induced morphological reversion was not observed with such structurally related molecules as DS-SG-45 ((2*S*,3*R*)-2-amino-3-hydroxy-4-(3-octylphenyl)butyl phosphoric acid) and DS-SG-12 ((2*S*,3*R*)-2-amino-4-(4-octylphenyl)butane-1,3-diol). The S1P- and DS-SG-44-induced shape changes were nseither reproduced with the $S1P_1$/$S1P_3$ receptor agonist VPC24191 nor inhibited by the $S1P_1$/$S1P_3$ receptor antagonist, VPC23019. Transfection with small interfering RNA (siRNA) for the $S1P_2$ receptor greatly inhibited the DS-SG-44-induced shape change, and in part an S1P-induced response. In the presence of VPC23019, siRNA transfection for the $S1P_2$ receptor almost completely blocked the S1P- and DS-SG-44-induced shape changes. Our results suggested that DS-SG-44, a newly-synthesized S1P analogue, acted as an S1P receptor agonist and that the S1P-induced shape change in rat C6 glioma cells was mediated mainly through the $S1P_2$ receptor, and cooperatively through the $S1P_1$/$S1P_3$ receptors.

Laboratory of Pharmacology, College of Pharmacy (BK21 Project) and Research Institute for Drug Development, Pusan National University, Busan 609-735, Republic of Korea

Kyeok Kim, Yu-Lee Kim, Santosh J. Sacket, Hyo-Lim Kim, Mijin Han, Dong-Soon Im

Department of Chemistry, Sogang University, Seoul 121-742, Republic of Korea

Deok S. Park, Baeck K. Lee, Won K. Lee

Department of Chemistry, Hankuk University of Foreign Studies, Yongin, Kyunggi-Do 449-719, Republic of Korea

Hyun-Joon Ha

Correspondence: D.-S. Im, Laboratory of Pharmacology, College of Pharmacy, Pusan National University, San 30, Jang-Jun-dong, Geum-Jung-gu, Busan 609-735, Republic of Korea. E-mail: imds@pusan.ac.kr

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Introduction

Sphingosine 1-phosphate (S1P) is highly abundant in and readily released from activated platelets (Yatomi et al 2000), and has been shown to be involved in a variety of cellular functions such as growth, differentiation and programmed death (Meyer zu Heringdorf et al 1997; Hla et al 2001). S1P functions as a first messenger through the G protein-coupled receptors, termed $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$ and $S1P_5$ (Lee et al 1998; Gonda et al 1999; Okamoto et al 1999; Im et al 2000; Van Brocklyn et al 2000; Yamazaki et al 2000), and as a second messenger through unclear mechanisms in certain cell types (Spiegel et al 1996). The G protein-couplings and intracellular signalling of each plasma membrane receptor has been investigated by using recombinant DNA such as $S1P_2$ (Gonda et al 1999; Van Brocklyn et al 1999). $S1P_2$ increased intracellular Ca^{2+} concentration via a Gq-PLC pathway and activated mitogen-activated protein (MAP) kinases in a pertussis toxin-sensitive manner (An et al 1999; Gonda et al 1999). Furthermore, $S1P_2$ induced cell rounding and a neurite retraction, presumably via $G_{12/13}$ (Van Brocklyn et al 1999).

Treatment of rat glioma C6 cells or astroglial cells with a β-adrenergic agonist such as isoprenaline resulted in a rapid increase in intracellular cAMP levels (Oey 1975; Shain et al 1987). In a serum-free medium, the cells subsequently changed from a fibroblast-like flat morphology to a more rounded astrocyte-like shape with defined processes (Tas & Koschel 1990, 1998). S1P, lysophosphatidic acid (LPA), and thrombin have been reported to induce morphological reversion from the isoprenaline-induced astrocyte-like shape to a fibroblast-like morphology through independent signalling pathways (Tas & Koschel 1990, 1998). The objectives of our study were to develop new drugs (S1P analogues), acting on S1P receptors and to find S1P receptor subtypes responsible for S1P-induced morphological

reversion in C6 glioma cells. We applied the cellular morphological response as a test of the agonistic effect of these S1P-related synthetic chemicals.

Materials and Methods

Materials

Sphingosine 1-phosphate (S1P), VPC23019 and VPC24191 were purchased from Avanti Polar Lipid (Alabaster, AL). All other materials were purchased from Sigma-Aldrich (St Louis, MO).

Synthesis of S1P analogues DS-SG-44 and DS-SG-45

Weinreb's amide **1** (Figure 1) was readily synthesized from enantiomerically pure aziridine-2(*S*)-mentholester (Lee & Ha 2003). Ketone **2a** and **2b** (Figure 1), a mixture of *cis* and *trans* olefins, was synthesized using Grignard reagent. The ketone was stereoselectively reduced by $ZnCl_2$ and $NaBH_4$ to give the corresponding alcohol **3a** and **3b** (Figure 1) at 99% (**3a**) and 96% (**3b**) yields (Yun et al 2003). The aziridine ring C(3)–N bond was regioselectively cleaved by treating with acetic acid (Lee et al 2001). Reduction of the double bond, debenzylation, and Boc protection of the amino group were accomplished by catalytic hydrogenation under H_2(g) 100 psi at a time with a 98% yield (Kim et al 2005). Dimethyl phosphate **6a** and **6b** (Figure 1) was synthesized by CBr_4 and dimethylphosphite under 0°C. Hydrolysis and *N*-Boc deprotection was accomplished by using TMS-Br and H_2O to give the corresponding sphingosine-1-phosphate analogues, DS-SG-44 and DS-SG-45 (Figures 1, 2) (Lim et al 2004).

All compounds were characterized by ^1H and ^{13}C NMR, and mass spectroscopy.

DS-SG-44

^1H NMR (300 MHz, $CDCl_3$) δ=7.24–7.15 (m, 4H), 4.49–4.42 (m, 1H), 4.29–4.20 (m, 1H), 4.13–4.08 (m, 1H), 3.45–3.41 (m, 2H), 2.86–2.82 (m, 2H), 2.61 (t, J=7.5 Hz, 2H), 1.64–1.58 (m, 2H), 1.40–1.26 (m, 12H), 0.93 (t, J=6.3 Hz, 3m) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ=141.4, 135.2, 129.3, 128.5, 76.0, 69.1, 61.5, 60.3, 40.0, 35.6, 32.4, 31.1, 28.4, 22.8, 14.2 ppm; HRMS: *m/z* calcd for $C_{18}H_{32}KNO_5P$ $[M+K]^+$ 412.1655, found 412.1671.

DS-SG-45

^1H NMR (300 MHz, $CDCl_3$) δ=7.13–6.93 (m, 4H), 4.36–4.29 (m, 1H), 4.17–4.08 (m, 1H), 4.04–3.98 (m, 1H), 3.34–3.30 (m, 1H), 2.78–2.65 (m, 1H), 2.48 (t, J=7.8 Hz, 2H), 1.54–1.47 (m, 2H), 1.30–1.12 (m, 12H), 0.76 (t, J=6.3 Hz, 3H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ=143.6, 138.0, 129.7, 128.7, 126.9, 126.7, 77.0, 70.1, 67.1, 67.0, 40.5, 36.1, 32.0, 31.7, 28.5, 22.8, 14.2 ppm; HRMS: *m/z* calcd for $C_{18}H_{32}KNO_5P$ $[M+K]^+$ 412.1655, found 412.1606.

Cell culture

Rat C6 glioma cells were maintained in high glucose Dulbecco's Modified Eagle's Medium (DMEM), containing 10% (v/v) fetal bovine serum, 100 U mL^{-1} penicillin, 50 μg mL^{-1} streptomycin, 2 mM glutamine, and 1 mM sodium pyruvate, at 37°C in a humidified 5% CO_2 incubator (Lee & Im 2006).

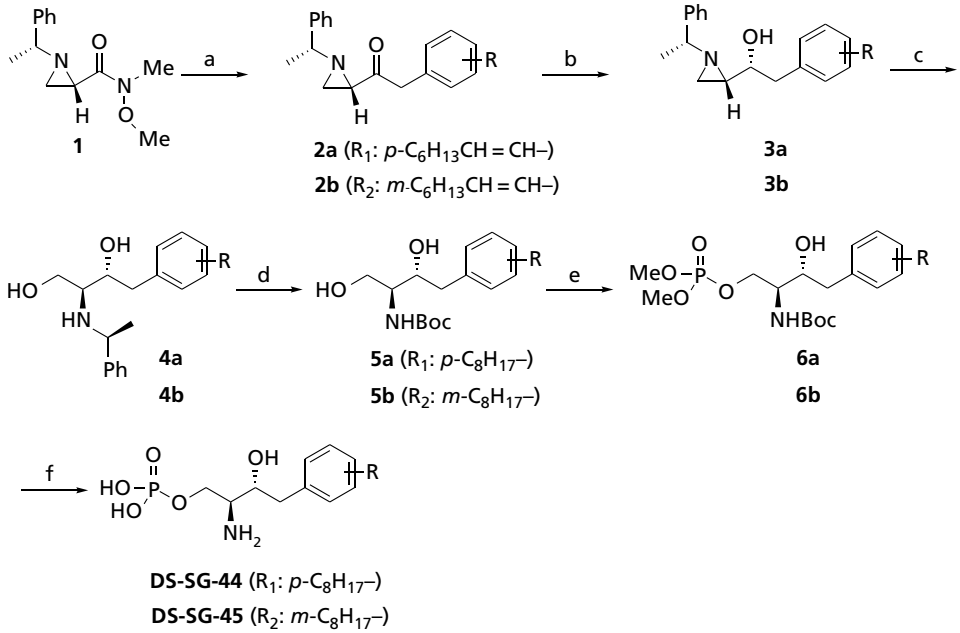

Figure 1 Synthesis of DS-SG-44, DS-SG-45 and other analogues. Reagents and conditions: a, Mg, 1-(bromomethyl)-4-(oct-1-enyl)benzene or 1-(bromomethyl)-3-(oct-1-enyl)benzene, THF, rt->reflux; b, $ZnCl_2$, MeOH, $NaBH_4$, −78°C; c, (i) AcOH, CH_2Cl_2, rt. (ii) KOH, EtOH, rt; d, $Pd(OH)_2$ 20 wt%, H_2(g) 100 psi, $(Boc)_2O$, rt; e, CBr_4, $P(OMe)_3$, Pyridine, 0°C; f, (i) TMS-Br, CH_2Cl_2, rt. (ii) H_2O.

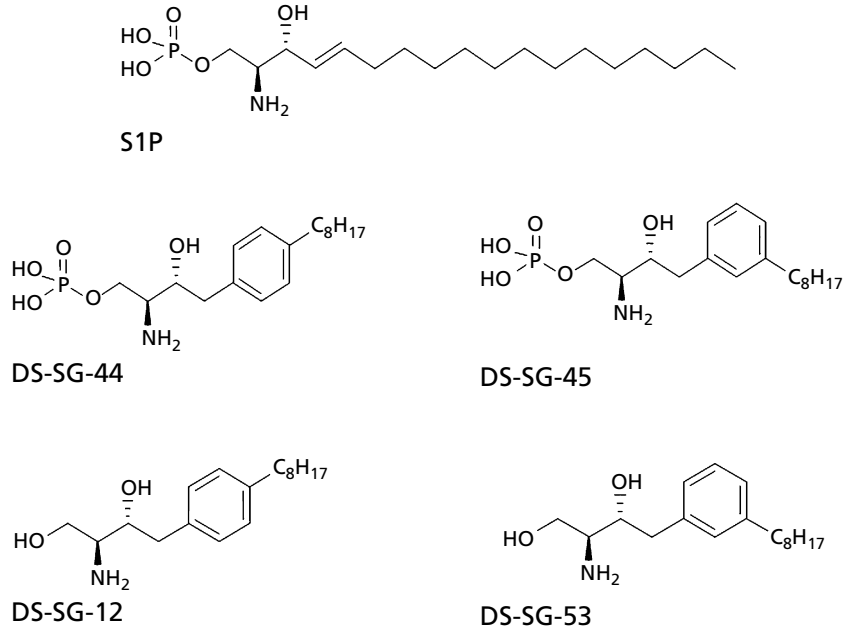

Figure 2 Structures of S1P, DS-SG-44 and other synthetic chemicals.

Morphological reversion test

The rat C6 glioma cells were seeded at low density on 12-well culture dishes and incubated overnight. The cells were then washed with 1 mL DMEM without serum, and 1 mL of the same medium added. Subsequently, 10 μL D, L-isoprenaline (stock solution 10^{-2} M) was added to yield a final concentration of 10^{-4} M and the cells were incubated at 37°C for 15 min.

To study the morphological changes induced by isoprenaline and their reversion by S1P or synthetic chemicals, vehicle, S1P (10 μM), DS-SG-44 (10 μM), DS-SG-45 (10 μM), DS-SG-12 (10 μM), or DS-SG-53 (10 μM) was then added and the cells incubated for an additional 15 min.

To study the morphological changes induced by isoprenaline, change reversion by S1P and the effects of various pharmacologic agents after isoprenaline treatment, vehicle, VPC24191 (30 μM), S1P (10 μM), premixture of S1P (10 μM) plus VPC23019 (30 μM), DS-SG-44 (10 μM), or a premixture of DS-SG-44 (10 μM) plus VPC23019 (30 μM) was then added and the cells incubated for an additional 15 min.

To assess the morphological changes and their reversion in $S1P_2$-siRNA transfected rat C6 glioma cells, after 24-h transfection of small interfering RNA (siRNA) for $S1P_2$ receptor, the cells were washed twice with serum-free DMEM and pre-incubated for 10 min at 37°C in serum-free DMEM. Cells were incubated for 15 min with 10^{-4} M D, L-isoprenaline followed by an additional 15-min incubation with vehicle, 10 μM S1P or 10 μM DS-SG-44. For the presence of 30 μM VPC23019, siRNA-transfected cells were treated with VPC23019 simultaneously when isoprenaline was added. Four images of cells from each well were obtained thereafter using an inverted microscope (TS-100, Nikon, Japan) and a digital camera (Coolpix 4500, Nikon, Japan). The experiment was conducted more than three times in different days. Total numbers of cells counted for each experiment were always over 100.

Design and transfection of siRNA

Rat C6 glioma cells were seeded on 12-well plates at 1.7×10^5 cells/well and cultured for 18 h before transfection. The incubation medium was changed to Opti-MEM and the siRNA for $S1P_2$ was transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following the instructions of the manufacturer. After 4-h incubation, medium was changed to Minimum Essential Medium (MEM) containing 10% fetal bovine serum. Two siRNA sequences for rat $S1P_2$ were designed and synthesized by the Samchuli Pharm Co. (Suwon, Korea). The following sequences were effective: sense 5′-UUA GCA UCC UUC UCU UAG ATT-3′ and antisense 5′-UCU AAG AGA AGG AUG CUA ATT-3′.

Statistical analysis

The results were expressed as mean ± s.e. of the number of determinations as indicated (Figures 3–5). Statistical significance of differences were determined by analysis of variance and significance was accepted when $P < 0.05$.

Results

S1P and DS-SG-44 induced shape changes in C6 rat glioma cells

Cytoplasm of the cells was observed to retract around the nucleus within 15 min after the addition of isoprenaline, and processes were formed at the margins (Figure 3B). When the

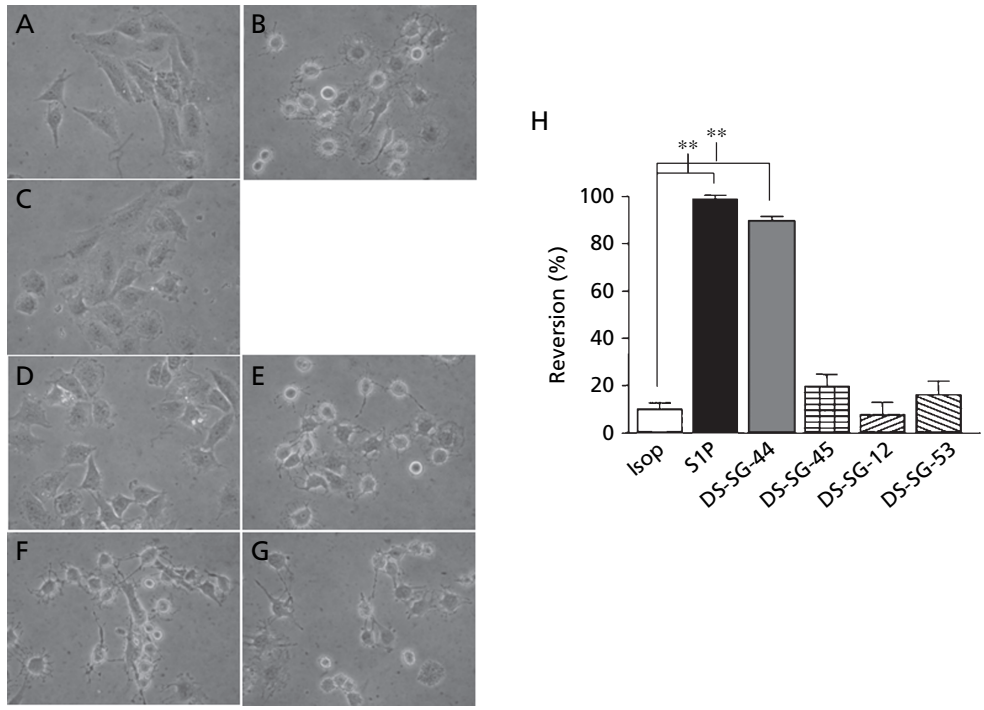

Figure 3 Morphological changes induced by isoprenaline and their reversion by S1P or synthetic chemicals. The rat C6 glioma cells were seeded at low density in 12-well dishes and allowed to stabilize for 24 h. Before the test incubations the cells were washed twice with serum-free DMEM and pre-incubated for 10 min at 37°C in serum-free DMEM. A. Control without additions. B. Cells incubated for 15 min with 10^{-4} M D, L-isoprenaline. Cells incubated for 15 min with 10^{-4} M D, L-isoprenaline followed by an additional 15-min incubation with 10 μM S1P (C), DS-SG-44 (D), DS-SG-45 (E), DS-SG-12 (F) or DS-SG-53 (G). H. Reversed round cells were counted and their percentage of the total presented as mean ± s.e. of three independent experiments on different days. $^{**}P < 0.01$, compared with the values obtained from the isoprenaline (Isop) only condition. Phase-contrast photographs ×400.

S1P was added, the morphological response was reversed by approximately 97% as reported by Tas & Koschel (1998) (Figure 3C and H). DS-SG-44 ((2*S*,3*R*)-2-amino-3-hydroxy-4-(4-octylphenyl)butyl phosphoric acid) induced a similar response as S1P (approximately 90%; Figure 3D), but this was not the case for DS-SG-45 ((2*S*,3*R*)-2-amino-3-hydroxy-4-(3-octylphenyl)butyl phosphoric acid) (Figure 3E), a compound with an octyl group at the *meta* position in contrast to the *para* position in DS-SG-44 (Figure 2). DS-SG-12 ((2*S*,3*R*)-2-amino-4-(4-octylphenyl)butane-1,3-diol) and DS-SG-53, which are structural analogues of DS-SG-44 and DS-SG-45 lacking a phosphate group (Figure 2), did not induce the response (Figure 3F and G), thereby strengthening the importance of the phosphate group for S1P receptor activation (Wang et al 2001).

Involvement of $S1P_1$, $S1P_2$ and $S1P_3$ receptors in the shape change response

To identify the S1P receptors responsible for the morphological response an $S1P_1/S1P_3$ receptor specific activator and inhibitor, VPC24191 and VPC23019, respectively, were used. As shown in Figure 4, the $S1P_1/S1P_3$ receptor specific activator, VPC24191, induced morphological change, but with less than 20% of the cells. Treatment with the $S1P_1/S1P_3$ receptor specific inhibitor, VPC23019, inhibited S1P- and DS-SG-44-induced morphological change very slightly, less than 10% (Figure 4). Statistically significant morphological changes were observed with the $S1P_1/S1P_3$ receptor agonist VPC24191 and antagonist VPC23019, however the degree was less than 20% (Figure 4), suggesting a minor role of the $S1P_1/S1P_3$ receptor in S1P-induced morphologic reversion in C6 glioma cells.

Small interference RNA (siRNA) technology was applied in an attempt to test the involvement of $S1P_2$ receptors in the response. Transfection with siRNA appeared to blunt the S1P- and DS-SG-44-induced morphological change (Figure 5). siRNA transfection for the $S1P_2$ receptor almost completely blocked the DS-SG-44-induced response (approximately 78%; Figure 5); however, the S1P-induced response was partially blocked (approximately 52%; Figure 5). The same experiment was repeated but in the presence of VPC23019, a specific inhibitor for the $S1P_1/S1P_3$ receptor. As shown in Figure 5, VPC23019 further inhibited the S1P-induced morphological change in the $S1P_2$-siRNA-transfected rat C6 glioma cells.

Discussion

We have identified an agonistic effect of DS-SG-44, a newly-synthesized S1P analogue, on S1P receptors and the role of the S1P receptors for shape changes of rat C6 glioma cells. Morphological reversion of the rat C6 glioma cells was employed as a screening method for the S1P receptor agonist,

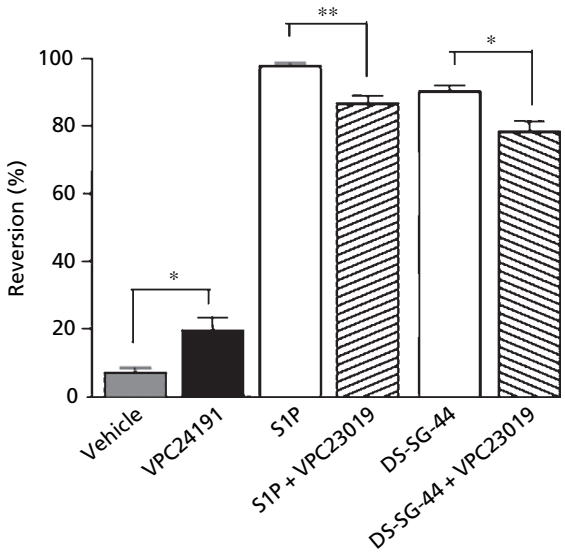

Figure 4 Morphological changes induced by isoprenaline, change reversion by S1P and the effects of various pharmacological agents. Cells incubated for 15 min with 10^{-4} M D, L-isoprenaline followed by an additional 15-min incubation with vehicle, VPC24191 (30 μM), S1P (10 μM), premixture of S1P (10 μM) plus VPC23019 (30 μM), DS-SG-44 (10 μM), or premixture of DS-SG-44 (10 μM) plus VPC23019 (30 μM). Reversed round cells were counted and percentages are presented as mean ± s.e. of three independent experiments on different days. $^*P < 0.05$, $^{**}P < 0.01$.

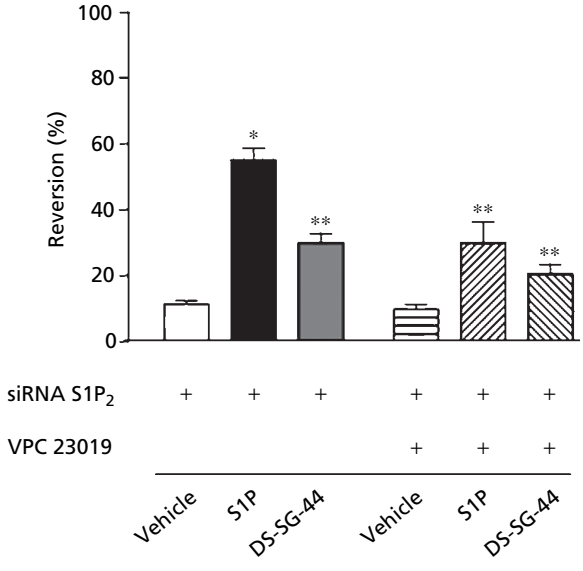

Figure 5 Morphological changes and their reversion in $S1P_2$-siRNA transfected rat C6 glioma cells. After 24-h transfection of siRNA for $S1P_2$ receptor, the cells were incubated for 15 min with 10^{-4} M D, L-isoprenaline followed by an additional 15-min incubation with vehicle, 10 μM S1P or 10 μM DS-SG-44. For the presence of 30 μM VPC23019, VPC23019 was added simultaneously when isoprenaline was added. Reversed round cells were counted and percentages are presented as mean ± s.e. of three independent experiments on different days. $^*P < 0.05$, $^{**}P < 0.01$ vs without siRNA transfection in Figure 3.

and by applying pharmacological inhibitor and siRNA technology, the roles of $S1P_2$ receptor and $S1P_1$/$S1P_3$ receptor in the response could be explained, mainly by the $S1P_2$ receptor, and cooperatively by the $S1P_1$/$S1P_3$ receptors. Our results provide a simple and useful screening method for the $S1P_2$ receptor and a fundamental information base to develop subtype-specific agonists.

DS-SG-44 has a structural similarity with FTY-720, which is the prototype of a new generation of immunomodulators. FTY-720 is the result of extensive chemical derivatization based on the natural product myriocin, isolated from the ascomycete *Isaria sinclairii* (Chiba et al 1998; Yanagawa et al 1998; Yuzawa et al 2000; Brinkmann & Lynch 2002; Im 2003). FTY-720 is effectively phosphorylated by sphingosine kinases in-vivo and the phosphorylated FTY-720-phosphate was reported as an agonist for $S1P_1$, $S1P_3$, $S1P_4$ and $S1P_5$ receptors, but not the $S1P_2$ receptor (Brinkmann et al 2002; Mandala et al 2002). Gene deletion and reverse pharmacology study have shown that FTY-720 acts at $S1P_1$ receptors on lymphocytes and the endothelium, thereby inhibiting the egress of T and B cells from secondary lymphoid organs in the blood and their recirculation to inflamed tissues (Matloubian et al 2004; Sanna et al 2004). In contrast to S1P-mimicking effects, functional antagonism against certain S1P-stimulated responses, such as T-cell chemotaxis and angiogenesis, have been demonstrated for FTY-720 (Graeler & Goetzl 2002; LaMontagne et al 2006). The antagonistic function of FTY-720 has been suggested to be due to internalization and partial degradation of S1P receptors (Gräler & Goetzl 2004; Kaneider et al 2004; Oo et al 2007). Applications of FTY-720 for several autoimmune diseases, multiple sclerosis, atherosclerosis, and cancer are under investigation and under clinical trials (Azuma et al 2002; Brinkmann & Lynch 2002; Kappos et al 2006; Keul et al 2007; Ng et al 2007; Nofer et al 2007). DS-SG-12 is the dephosphorylated form of DS-SG-44. FTY-720 has an additional hydroxymethyl group on C2 and no hydroxyl group on C3 compared with DS-SG-12 (Brinkmann & Lynch 2002). FTY-720 and DS-SG-44 have a *para*-octyl-substituted phenyl structure instead of the long aliphatic chain of sphingosine. DS-SG-44 is supposedly an agonist for the $S1P_2$ receptor, while its effects on other types of S1P receptors remain to be elucidated. Further investigations on the metabolism of DS-SG-12 and effects of DS-SG-44 on the immune system in-vivo would be favoured. Our findings suggested the basis for future development of S1P receptor subtype-selective agonists.

In summary, we synthesized an S1P analogue, DS-SG-44, which acted as an S1P receptor agonist, and found that the S1P-induced shape change in rat C6 glioma cells was mediated mainly through the $S1P_2$ receptor and cooperatively through the $S1P_1$/$S1P_3$ receptors.

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