A Novel Sphingosine-1-Phosphate Receptor 1 Antagonist Prevents the Proliferation and Relaxation of Vascular Endothelial Cells by Sphingosine-1-Phosphate

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A sphingosine-1-phosphate receptor 1 (SIP1) antagonist is expected to be an anti-angiogenic compound; however, there are few reports that demonstrated that a SIP1 inhibitor improved the disease state in an angiogenic animal model. Since we determined that a prototype SIP1 antagonist was an in vivo angiogenesis inhibitor, we developed the derivatives to acquire more effective compounds. In this report, we show the SIP1 antagonistic activity of some representatives, especially compound 5 {sodium 4-[(4-butoxyphenyl)thio]-2′-[4-[(heptythio)methyl]-2-hydroxyphenyl](hydroxy)methyl]biphenyl-3-sulfonate}. The IC50 values calculated from an intracellular cyclic AMP measurement assay and a [33P]sphingosine-1-phosphate (Sph-1-P)/SIP1 binding assay were 38 and 200 nm, respectively. A subtype specificity test for the other Sph-1-P receptors showed that compound 5 was the SIP1, directional antagonist. It also inhibited the proliferation, migration, and tube formation of human umbilical vein endothelial cells stimulated by Sph-1-P with the IC50 values of 18, 650, and 230 nm, respectively. A cytotoxicity assay concurrently performed with a tube formation assay supported the hypothesis that these biological effects were not due to its cytotoxicity. Furthermore, administration (10 mg/kg, intravenously) to anesthetized Sprague-Dawley rats inhibited Sph-1-P-induced hypotension by 100—90% for 30 min. This is presumably through the inhibition of Sph-1-P-induced vasorelaxation, mainly by the blocking of SIP1 and/or SIP3. Taken together, these results show that compound 5 is an inhibitor of in vitro and in vivo Sph-1-P signaling, and that it will be useful to elucidate the in vivo effect of Sph-1-P on vascular endothelial cells.

Key words sphingosine-1-phosphate; human umbilical vein endothelial cell; hypotension; sphingosine-1-phosphate receptor 1 antagonist

Sphingosine-1-phosphate (Sph-1-P) is a bioactive plasma lipid which affects cell proliferation and migration, etc., through 5 receptors: sphingosine-1-phosphate receptors 1 (SIP1) to 5 (SIP5).1) Among these receptors, SIP1 is the most studied and attractive pharmacological target because the SIP1 agonism provokes lymphopenia2) and immunosuppression in vivo. Meanwhile, SIP1 antagonism is supposed to inhibit angiogenesis.3,4) In the case of agonist compounds, the non-selective Sph-1-P receptor agonist FTY720 ( fingolimod, Novartis International AG, Basel, Switzerland) is being evaluated as a drug of multiple sclerosis. Additionally, the SIP1 specific agonist R-3477 (Actelion Pharmaceuticals Ltd., Basel, Switzerland) is being evaluated in Phase II and I studies as an immunosuppressant for autoimmune disease and organ transplantation, respectively. In the case of antagonist compounds, a preclinical report showed that the small interfering RNA for SIP1 inhibited tumor growth5); nevertheless, there have been no clinical reports on a chemical compound for the treatment of angiogenic diseases, such as diabetic retinopathy and solid tumors. This is presumably because there are few SIP1 antagonists that are potent and selective in vivo.

Both SIP1 and SIP3 also play a role in cardiovascular regulation. The intravenous (i.v.) administration of Sph-1-P at high doses of 100—200 μg/kg caused bradycardia in anesthetized rats.6,7) Moreover, the heart rate of SIP3 homozygous knockout mice was unchanged by Sph-1-P administration.7) These results indicate that Sph-1-P caused bradycardia via SIP1 in the heart, at least in the case of mice. On the other hand, i.v. administration of Sph-1-P at a low dose of 10 μg/kg provoked transient hypotension without affecting the heart rate in anesthetized rats.6) In this case, it is expected that Sph-1-P caused vasodilation on the vascular endothelial cells via SIP1 and SIP3, followed by activation of the endothelial isoform of nitric oxide synthase and NO production.8—11)

We previously reported that a SIP1 antagonist, designated as chemical lead 2, showed anti-angiogenesis activity in a rabbit cornea model and inhibition of the swelling of mouse feet in an anti-type II collagen antibody-induced arthritis model.12) Afterwards, we synthesized derivatives of chemical lead 2 to obtain more effective SIP1 antagonists and determined the representatives, named as compounds 1 to 5. The aim of this study is to elucidate whether the derivatives inhibit the effect of Sph-1-P both in vitro and in vivo. Therefore, here we describe the Sph-1-P antagonistic activity of the representative derivatives, compounds 1 to 5, through several in vitro and in vivo assays focusing on the proliferation, migration, tube formation, and relaxation of vascular endothelial cells.

MATERIALS AND METHODS

General Reagents and Animals Compound 1 {sodium 4-[(4-butoxyphenyl)thio]-2′-(1-hydroxytridec-2-yn-1-yl]-biphenyl-3-sulfonate}, compound 2 {sodium 4-[(4-butoxyphenyl)thio]-2′-[4-(hydroxy(2-hydroxy-4-nonanoyl-phenyl)methyl]biphenyl-3-sulfonate}, compound 3 {sodium 4-[(4-butoxyphenyl)thio]-2′-[4-[(heptyloxy)methyl]-2-(hydroxy(2-hydroxy-4-nonanoyl-phenyl)methyl]biphenyl-3-sulfonate}, compound 4-5 {sodium 4-[(heptyloxy)methyl]-2-(hydroxy(2-hydroxy-4-nonanoyl-phenyl)methyl]biphenyl-3-sulfonate}, compound 5 {sodium 4-[(4-butoxyphenyl)thio]-2′-[4-[(heptyloxy)methyl]-2-(hydroxy(2-hydroxy-4-nonanoyl-phenyl)methyl]biphenyl-3-sulfonate}.

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droxymethyl]phenyl]([hydroxy]methyl)biphenyl-3-sulfonate], compound 4 {sodium 4-[(4-butoxyphenyl)thio]-2-[(4-[(heptylthio)methyl]-2-hydroxyphenyl)](hydroxy) methyl}biphenyl-3-sulfonate}, and compound 5 {sodium 4-[(4-butoxyphenyl)thio]-2-[(4-[(heptylthio)methyl]-2-hydroxyphenyl)](hydroxy)methyl}biphenyl-3-sulfonate}, were synthesized in Exploratory Chemistry Research Laboratories, Sankyo Co., Ltd. (former company name of Daici Sankyo Co., Ltd. before the merger, Tokyo, Japan). Human umbilical vein endothelial cells (HUVEC), HuMedia-EG2 medium, and HuMedia-EB2 medium were purchased from Kurabo Industries Ltd. (Osaka, Japan). Other cell culture media and antibiotics were purchased from Invitrogen Corp. (Carlsbad, CA, U.S.A.) unless otherwise stated. Sph-1-P was purchased from BIOMOL International, L.P. (Plymouth Meeting, PA, U.S.A.). Inactin® (thiobutabarbital sodium salt) was purchased from Sigma-Aldrich Japan (Tokyo, Japan) unless otherwise stated. Male Sprague-Dawley rats (250—300 g) were purchased from Japan SLC, Inc. (Shizuoka, Japan). The animals were maintained in a room controlled at a temperature and relative humidity of 24±2°C and 40—70%, respectively, and given food and water ad libitum. The experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of Sankyo Co., Ltd.

**Primary Cells and Each Sph-1-P Receptor Expressing Cell** Chinese hamster ovary (CHO) cells that stably express human S1P₁—₄, namely CHO-S1P₁₋₄, were established previously.¹²—¹⁴) HUVEC were maintained with HuMedia-EG2 medium. CHO-S1P₁—₄ cells were cultured with α-Minimum Essential Medium containing 10% (v/v), diazoyl fetal bovine serum (SAFC Biosciences, Lenexa, KS, U.S.A.), 100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and 125 nm methotrexate without ribonucleosides and deoxyribonucleosides. CHO-S1P₂₋₄ cells were maintained with F-12 medium containing 10% (v/v) fetal bovine serum, 100 μg/ml penicillin G sodium, 10 μg/ml streptomycin sulfate, and 400 μg/ml G418.

For the measurement of S1P₁₋₄ signaling using a fluorometric imaging plate reader (FLIPR), CHO-S1P₁–Gqi5 cells and CHO-S1P₂–Gqi5 cells that contain Gqi5 chimeric G proteins were used instead of the original cells to enable the measurement of Ca²⁺ signaling.¹³) These cells were maintained in the suitable medium mentioned above with 200 μg/ml Hygromycin. For the measurement of S1P₂ and S1P₃ signaling, intact CHO-S1P₂ cells and CHO-S1P₃ cells were used, respectively.

**Cyclic AMP (cAMP) Assay and [³²P]Sph-1-P/S1P₁ Binding Assay** The cAMP assay and the binding assay using CHO-S1P₁ cells were performed as described previously.¹²,¹⁵) The 100 nm Sph-1-P was used as a stimulant in the cAMP assay. The specific activity of [³²P]Sph-1-P was 3400 cpm/fmol.

**Sph-1-P Receptor Subtype Specificity of Compound 5** The receptor subtype specificity of compound 5 was determined with a FLIPR system according to our previous report.¹⁴) For the measurement of Ca²⁺ signaling in CHO-S1P₁₋₄ cells, 100 nm, 100 μm, 10 μm, and 10 μM Sph-1-P were used as a stimulant, respectively, according to each cellular reactivity.

**HUVEC [⁶-³²P]Thymidine Uptake Assay** A [⁶-³²P]-thymidine uptake assay was performed according to the method of Wang et al. with modification.¹⁶) HUVEC were suspended in HuMedia-EB2 medium containing 1% (v/v) fetal bovine serum, 1 μg/ml hydrocortisone, 50 μg/ml gentamicin, 50 ng/ml amphotericin B, and 10 μg/ml heparin; and then 2500 of the cells were added to 96-well plates and cultured overnight. Dimethyl sulfoxide or a serial concentration of compound 5 was mixed into each well (n=6), and the HUVEC were stimulated with vehicle [80% (v/v) ethanol] or 100 nm Sph-1-P for 24 h at 37°C in 5% CO₂. Then, 0.5 μCi of [⁶-³²P]thymidine (GE Healthcare Bio-Sciences KK, Tokyo, Japan) was added to each well, and the HUVEC were further incubated for 7 h at 37°C in 5% CO₂. The cells were harvested onto Printed Filtermat A filters (WALLAC, Turku, Finland) with a Mach II cell harvester (Tomtec Inc., Hamden, CT, U.S.A.). The remaining cells in each well were detached with 100 μl of 0.25% (w/v) trypsin and reharvested onto the same filters. The radioactivity from the cells on the filters was measured with a BETA-PLATE (PerkinElmer Inc., Waltham, MA, U.S.A.).

**HUVEC Migration Assay** The inhibitory effect of compound 5 in the HUVEC migration assay was evaluated as described previously.¹³) The 100 nm Sph-1-P was used for a stimulant of migration.

**Rac Activation Assay** The activation state of Rac within Sph-1-P-stimulated HUVEC was examined using a Rac Activation Assay Kit (Upstate Biotechnology Inc., Charlottesville, VA, U.S.A.), following the instructions of the manufacturer. First, 1×10⁵ of HUVEC were seeded into 10 cm dishes with HuMedia-EG2 medium, cultured for 24 h at 37°C in 5% CO₂, and starved overnight in serum-free medium (HuMedia-EB2 medium containing 1 μg/ml hydrocortisone, 50 μg/ml gentamicin, 50 ng/ml amphotericin B, and 10 μg/ml heparin). On the day of the assay, dimethyl sulfoxide or 7.1 μM of compound 5 was mixed into the dishes and the HUVEC were stimulated for just 3 min at room temperature with vehicle [80% (v/v) ethanol] or 10 nm Sph-1-P. All of the medium was removed, the HUVEC were lysed with 0.8 ml of the ice-cold 1×MLB which came with the kit, and were placed on ice. The lysates were collected, the debris was removed by centrifugation, and 700 μl of supernatant of supernatant 10 μl of the PAC-1 PBD agarose beads which came with the kit were mixed and rotated for 1 h at 4°C. The beads were washed 3 times with 0.5 ml of 1×MLB, resuspended in 40 μl of 2×Laemmli sample buffer (Bio- Rad Laboratories, Hercules, CA, U.S.A.) containing 5% (v/v) 2-mercaptoethanol, and boiled for 5 min. Each boiled sample containing 5.7 μg of protein was loaded on a polyacrylamide gel. The HUVEC lysate containing 3 μg of protein was also loaded as a positive control. The activated Rac (beads-bound Rac) and the total Rac in HUVEC lysate were detected by immunoblotting with the anti-Rac mouse monoclonal antibody which came with the kit.

**HUVEC Tube Formation and Cytotoxicity Assay** A tube formation assay was performed according to the method of Lee et al. with modification.¹⁷) Cultured HUVEC were stripped by trypsinization, washed once, and resuspended with serum-free Medium 199. Then, 400 μl of 5×10⁴ cells/ml suspension was added to each well of BD BioCoat® Cellware Matrigel® Basement Membrane Matrix 48-well Plates (BD Biosciences, San Jose, CA, U.S.A.). The indi-
cated concentration of compound 5 or dimethyl sulfoxide and 40 μl of AlamarBlue (BioSource, Camarillo, CA, U.S.A.) were added to each well (n=4) and mixed. One micromolar Sph-1-P or vehicle [80% (v/v) ethanol] was added and the 48-well plate was incubated for 3 h at 37 °C in 5% CO₂. The cultured medium and plate were used for the cytotoxicity and tube formation assays, respectively.

For the HUVEC cytotoxicity assay, 200 μl of each cultured medium was moved to a 96-well flat clear bottom plate, and the fluorescence intensity of the reduced AlamarBlue (excitation at 530 nm/emission at 590 nm) in each cultured medium was measured with an AR VO SX (WALLAC, Turku, Finland) and a cardiotachometer (AT-601G, Nihon Kohden), respectively, and recorded on a heat-stable oscillograph (RJG-4128, Nihon Kohden). After stabilization, the Sph-1-P administration and the tested compound administration to the right and left femoral veins were cannulated for the measurement of Ca²⁺ concentration. One mouse was used for compound 2, compound 5 also measured in a 0.5 nM [³²P]Sph-1-P/S1P₁ binding assay and the IC₅₀ value was 200 nM. Next, we examined the Sph-1-P receptor subtype specificity of compound 5 through the FLIPR system using CHO-S1P₁α,β,γ cells. As shown in Table 2, compound 5 was the S1P₁-directional antagonist, although the antagonistic effect on other families of G protein-coupled receptor was not tested.

**Inhibitory Effect of Compound 5 on Both the Proliferation and Migration of Sph-1-P-Stimulated HUVEC** In the process of angiogenesis, Sph-1-P is thought to promote the proliferation, migration, and tube formation of vascular endothelial cells and to contribute to the extension of new blood vessels.³,16,17) HUVEC are convenient primary cells because Sph-1-P-stimulated HUVEC can reproduce all three steps necessary for angiogenesis, i.e., proliferation, migration, and tube formation. The inhibition (% of each compound against Sph-1-P-inhibited hypotension, whereas the minimum blood pressure after control injection of Sph-1-P was set at 0% inhibition. One mouse was used for compound 2, and two mice were used for compounds 1, 3, 4, and 5, respectively. In the case of using a pertussis toxin (PTX), 10 μg/kg PTX was i.p. administered once a day 3 d before the test. For the measurement of dose-dependency of compound 5, it was administered at a dose of 0.1, 1, or 10 mg/kg; and Sph-1-P trials were performed at 3, 6, 9, 12, 15, 20, 25, and 30 min after compound administration. All the drugs were dissolved in 0.9% (w/v) saline solution. The inhibition (%) of each compound against Sph-1-P-induced hypotension was calculated from the minimum blood pressure after each Sph-1-P trial and plotted. The average of minimum blood pressure in stable state was set at 100% inhibition, whereas the minimum blood pressure after control injection of Sph-1-P was set at 0% inhibition. One mouse was used for compound 2, and two mice were used for compounds 1, 3, 4, and 5, respectively. In the case of using a pertussis toxin (PTX), 10 μg/kg PTX was i.p. administered once a day 3 d before the test. For the measurement of dose-dependency of compound 5, it was administered at a dose of 0.1, 1, or 10 mg/kg; and Sph-1-P trials were performed at 3, 6, 9, 12, 15, 20, 25, 30, 40, 50, and 60 min after compound administration (n=3).

**Statistical Analysis** The statistical significance of the differences between the mean values was calculated by a non-paired t-test. A p value of <0.05 was considered to be significant. The IC₅₀ values of compounds were estimated with Prism 4 software (GraphPad Software, Inc., La Jolla, CA, U.S.A.).

**RESULTS**

**SIP₃ Antagonistic Activity of Compounds 1—5 and the**

Sph-1-P Receptor Subtype Specificity of Compound 5 Compounds 1—5 are representative derivatives of a prototype S1P₁ antagonist, chemical lead 2. Therefore, we first checked the S1P₁ antagonistic activity of compounds 1—5 using CHO-S1P₁ cells. As shown in Table 1, compounds 1—5 inhibited the intracellular cAMP concentration decrease induced by 100 nM Sph-1-P, and the IC₅₀ values were 200, 220, 54, 49, and 38 nM, respectively. The S1P₁ antagonistic activity of compound 5, which was the most potent inhibitor, was also measured in a 0.5 nM [³²P]Sph-1-P/S1P₁ binding assay and the IC₅₀ value was 200 nM. Next, we examined the Sph-1-P receptor subtype specificity of compound 5 through the FLIPR system using CHO-S1P₁α,β,γ cells. As shown in Table 2, compound 5 was the S1P₁-directional antagonist, although the antagonistic effect on other families of G protein-coupled receptor was not tested.

**Table 1. S1P₁ Antagonistic Activities of Compounds 1—5, Which Were Measured in a cAMP Assay**

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>IC₅₀ (nM)</th>
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<tbody>
<tr>
<td>1</td>
<td></td>
<td>200</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>220</td>
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<td>3</td>
<td></td>
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</tr>
<tr>
<td>4</td>
<td></td>
<td>49</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>38</td>
</tr>
</tbody>
</table>

The IC₅₀ value was calculated from the inhibition (%) of cAMP concentration decrease in S1P₁-expressing cells, which were stimulated with 100 nM of Sph-1-P.

**Table 2. The Sph-1-P Receptor Subtype Specificity of Compound 5**

<table>
<thead>
<tr>
<th>SIP₁</th>
<th>SIP₂</th>
<th>SIP₃</th>
<th>SIP₄</th>
<th>SIP₅</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.6</td>
<td>3</td>
<td>14</td>
<td>n.t.</td>
<td></td>
</tr>
</tbody>
</table>

The IC₅₀ value was calculated from the inhibition (%) of the Ca²⁺ signaling of each receptor, which was measured with a fluorometer imaging plate reader (FLIPR). For the measurement of Ca²⁺ signaling in CHO-S1P₁ α,β,γ cells, the 0.1 nM, 1 nM, 10 nM, and 10 μM Sph-1-P were used as a stimulant, respectively, according to each cellular reactivity. ---, no inhibition; n.t., not tested.
tion, and tube formation. Moreover, as HUVEC express some members of Sph-1-P receptor, it is critical to determine whether compound 5 inhibits these three steps or not. As shown in Table 3, compound 5 inhibited [6-3H]thymidine uptake of Sph-1-P-stimulated HUVEC, and the IC50 value was 18 nM. This finding indicated that compound 5 suppressed DNA synthesis and the proliferation of HUVEC by antagonizing Sph-1-P receptors, presumably S1P1. Compound 5 also inhibited Sph-1-P-induced HUVEC migration with an IC50 value of 650 nM (Table 3). In addition, we checked the effect of compound 5 on the Rac activation of Sph-1-P-stimulated HUVEC because Rac, a Rho family small GTPase, is an essential regulator of Sph-1-P-induced HUVEC migration. The 3 min stimulation of serum-starved HUVEC with 10 nM Sph-1-P activated the Rac, and 7.1 μM of compound 5 completely negated the effect of Sph-1-P (Fig. 1). These results suggest that compound 5 blocked the Sph-1-P receptors and interrupted the Sph-1-P signaling for the proliferation and migration of vascular endothelial cells.

Inhibitory Effect of Compound 5 on HUVEC Tube Formation

Next, we confirmed the effect of compound 5 on HUVEC tube formation. In order to exclude the possibility that the compound’s toxicity itself brings about the inhibition of tube formation, we concurrently performed a HUVEC tube formation assay and a cytotoxicity assay with the same 48-well plate. After 3 h incubation with or without 1 μM Sph-1-P and various concentrations of compound 5, the conditioned media were used for the cytotoxicity assay and the tubular HUVEC in the same wells were fixed and stained for quantification of the total tube length. Figures 2A—C shows that compound 5 concentration-dependently inhibited the Sph-1-P-provoked HUVEC tube formation with an IC50 value of 230 nM. In the cytotoxicity assay, 1 μM Sph-1-P activated HUVEC metabolism and elevated the fluorescence intensity of reduced AlamarBlue from 52142 ± 2338 to 72279 ± 4875 (Fig. 2D, the right-end and left-end bars, respectively). Compound 5 concentration-dependently inhibited the increase of the fluorescence intensity of Sph-1-P-stimulated HUVEC, but the intensity was never below that of unstimulated HUVEC, at least up to 14.2 μM (Fig. 2D). These data clearly show that the inhibitory effect of compound 5 on tube formation is based on a blockade of the Sph-1-P receptors and is not due to the compound’s cytotoxicity.

Table 3. The Inhibitory Effect of Compound 5 on [6-3H]Thymidine Uptake and Migration Assay Using HUVEC

<table>
<thead>
<tr>
<th>Thymidine uptake</th>
<th>Migration</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC50 (nM)</td>
<td>18</td>
</tr>
</tbody>
</table>

The 100 nM Sph-1-P was used as a stimulant in both assays.

Fig. 1. Rac Activation Assay

Each compound was added to serum-starved HUVEC, then HUVEC were stimulated with or without 10 nM Sph-1-P for 3 min. The activated Rac and the total Rac in HUVEC lysate were detected by immunoblotting. Lane no. 1, dimethyl sulfoxide + 80% (v/v) ethanol; no. 2, dimethyl sulfoxide + 10 nM Sph-1-P; no. 5, 7.1 μM compound 5 + 10 nM Sph-1-P; no. 10, HUVEC lysate; others, unrelated compound + 10 nM Sph-1-P.

Fig. 2. HUVEC Tube Formation and Cytotoxicity Assay

(A, B) Representative photographs of HUVEC tube formation induced by 1 μM Sph-1-P. Dimethyl sulfoxide (A) or when 0.142 μM of compound 5 (B) was added. Each magnification = ×400. (C) Quantification of tube formation. HUVEC tube formation under various concentrations of compound 5 was quantified as the total tube length. Data represent the means ± S.D. (n=4). IC50 value of compound 5 = 0.23 μM. ∗p<0.05 vs. without a compound and Sph-1-P. (D) HUVEC cytotoxicity assay. At the end of the tube formation assay, each cultured medium in the 48-well plate was moved into a new 96-well plate, and the fluorescence intensity of the reduced AlamarBlue (excitation at 530 nm/emission at 590 nm) in each cultured medium was measured with an ARVO SX as the index of HUVEC metabolism. Data represent the means ± S.D. (n=4). The inhibitory effect of compound 5 is not due to cytotoxicity. ∗p<0.05 vs. without a compound and Sph-1-P.
Inhibitory Effect of Compounds 1—5 on the Sph-1-P-Induced Hypotension

Finally, we tested the antagonistic activity of compounds 1—5 in vivo. Since Sugiyama et al. showed that i.v. injection of Sph-1-P at a dose of 10 µg/kg brought about a transient decrease of mean blood pressure for 30 s without affecting the heart rate in anesthetized rats, we followed the same procedure. The i.v. injection of 10 µg/kg Sph-1-P caused transient hypotension for 40 s and did not affect the heart rate in anesthetized Sprague–Dawley rats (Fig. 3A). I.p. pre-administration of 10 µg/kg PTX for 3 d eliminated the effect of Sph-1-P, which indicated this hypotension response was mediated by Gi-coupled receptor, probably S1P1 and/or S1P3 (Fig. 3A). As shown in Fig. 3B, compounds 1, 3, and 4 (each 10 mg/kg) inhibited the Sph-1-P-induced hypotension at 3 min, but these antagonistic activities were gradually attenuated. Compound 2 did not show antagonistic activity. On the other hand, compound 5 perfectly prevented hypotension. Figure 3C shows the time course of the antagonistic activity of all the compounds. It was noteworthy that compound 5 completely inhibited Sph-1-P-induced hypotension over 30 min. The dose-dependency test of compound 5 revealed that 10 mg/kg administration of compound 5 showed good reproducibility and it inhibited the hypotension by 100—90% for 30 min (Fig. 3D). These results show that compound 5 has potent antagonistic activity against in vivo Sph-1-P signaling.

DISCUSSION

By derivatization, S1P1 antagonist activity of compound 5 as the IC50 value in the cAMP assay improved and was 150-fold stronger than that of chemical lead 2, from 5.7 µM to 38 nM (Table 1). Additionally, in the binding assay, the IC50 value also improved 125-fold, from 25 µM to 200 nM. On the other hand, the S1P1 specificity of compound 5 among Sph-1-P receptors did not dramatically improve (Table 2). Although there is some difference between the IC50 values in the cAMP and [33P]Sph-1-P/S1P1 binding assay (38, 200 nM, respectively), this is possibly due to the influence of fatty acid-free bovine serum albumin in the binding assay buffer. The albumin is necessary to suppress the non-specific binding of [33P]Sph-1-P, but it also reduces the antagonistic activity of compound 5. The S1P1 antagonist activity of compound 5 measured with FLIPR was weaker than that in the

Fig. 3. The Sph-1-P-Competitive Effect of i.v. Pre-administered Compounds 1—5 in Anesthetized Rats

The whole body blood pressure of each anesthetized Sprague-Dawley rat was recorded. (A) The i.v. injection of 10 µg/kg Sph-1-P (black arrow) caused transient hypotension for 40 s but hardly affected the heart rate (middle and upper charts, respectively). I.p. pre-administration of 10 µg/kg pertussis toxin (PTX) for 3 d eliminated the effect of Sph-1-P (bottom chart). (B) The comparison of antagonistic effect of compounds. Ten mg/kg of each compound was i.v. administered at 0 min, then 10 µg/kg Sph-1-P was i.v. administered repeatedly (black arrows). Sph-1-P-induced hypotension reappeared time-dependently in compound 1, 3, and 4 administered rats, whereas the hypotension never appeared for 12 min in compound 5-administered rats. Compound 2 did not show the antagonistic activity in this test. (C) The inhibitory effect of compounds 1—5 on Sph-1-P-induced hypotension. Ten milligrams per kilograms of each compound was i.v. administered at 0 min, then 10 µg/kg Sph-1-P was i.v. administered repeatedly at the indicated time points (compound 2, n=1; others, n=2). The inhibition (%) of each compound against Sph-1-P-induced hypotension was calculated from the minimum blood pressure after each Sph-1-P injection and plotted. The average of minimum blood pressure in a stable state was set at 100% inhibition, whereas the minimum blood pressure after control injection of Sph-1-P was set at 0% inhibition. (D) The dose-dependency of compound 5 on the hypotension test. The 0.1, 1, or 10 mg/kg of compound 5 was i.v. administered. The same experimental and calculation methods described above were used (n=3).
cAMP assay (Tables 1, 2). Not only this compound, but also most derivatives, the IC_{50} values against S1P, measured with FLIPR tended to be one-figure higher than those in the cAMP assay (data not shown). This may be the influence of Gq5 chimeric G protein, which is used in FLIPR assay and converts S1P, downstream signaling from cAMP to Ca^{2+}.

Compound 5 inhibited S1P, the most, with S1P, being the next most inhibited among the Sph-1-P receptors (Table 2). This offers an advantageous opportunity to study the function of Sph-1-P on vascular endothelial cells because both S1P, and S1P, especially S1P, are supposed to be important for both the response of vascular endothelial cells, such as HUVEC and neovascularization by Sph-1-P. Indeed, compound 5 could inhibit the proliferation, migration, and tube formation of Sph-1-P-stimulated HUVEC (Table 3, Fig. 2). Although a higher concentration of compound 5 was needed compared to S1P, expressing cells, this may be due to HUVEC express multiple Sph-1-P receptors. In these three assays, the incubation time of compound 5 with HUVEC was 31, 4, and 3 h, respectively. Longer incubation in [6-3H]thymidine uptake assay may bring about a stronger inhibitory effect than the other two assays. In addition, because the incubation time for the tube formation assay is 3 h, it is thought that migration rather than proliferation is important for the formation of the HUVEC capillary network. Therefore, it is a reasonable result that IC_{50} values in the migration and tube formation assay (650, 230 nM, respectively) were close.

Compound 5 inhibited Sph-1-P-induced transient hypotension in Sprague-Dawley rats (Figs. 3C, D). A previous report showed that Sph-1-P dose-dependently induced transient relaxation of rat mesenteric arteries via activation of the endothelial isofrom of nitric oxide synthase with an EC_{50} value of 10 ± 3 nM. Therefore, in our rat study, the i.v. injection of Sph-1-P probably caused direct vasorelaxation and resulted in transient hypotension. Taking these findings together, it was demonstrated that compound 5 prevented Sph-1-P-induced vasorelaxation and hypotension mainly by blocking S1P, and/or S1P, on the vascular endothelial cells of the rats, although the precise mechanism must be investigated in the future. There are some differences of the inhibitory effect of compounds in the cAMP assay and the hypotension test. The IC_{50} values of compounds 1 and 2 in the cAMP assay were equivalent (200, 220 nM, respectively); however, compound 2 was ineffective in the hypotension test (Fig. 3C). Similarly, the IC_{50} values of compounds 3, 4, and 5 in the cAMP assay were also equivalent (54, 49, 38 nM, respectively); however, compound 5 showed strong inhibition in the hypotension test (Figs. 3C, D). The most possible reason is the difference of solubility of compounds. Compounds 1 and 5 are easily dissolved in distilled water, but the solubility of other compounds is inferior. It may cause the difference of plasma concentration of compounds. Another possible reason might be the difference of the stability of compounds in rat plasma.

In conclusion, the S1P, directed antagonist compound 5 inhibited the biological responses of HUVEC induced by Sph-1-P, i.e., proliferation, migration, and tube formation. Moreover, it showed a potent inhibitory effect on Sph-1-P-induced hypotension in rats. Compound 5 will be a useful tool to analyze the in vivo effect of Sph-1-P, especially on vascular endothelial cells.

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