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Title page

Sphingosine 1-Phosphate Regulates Vascular Contraction via S1P₃ Receptor:
Investigation Based on a New S1P₃ Receptor Antagonist

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Abstract; 220 words, Introduction; 547 words, Discussion; 1152 words

d) Abbreviations:

S1P, sphingosine 1-phosphate; GPCR, G protein-coupled receptor; TY-52156,
1-(4-chlorophenylhydrazono)-1-(4-chlorophenylamino)-3,3-dimethyl-2-butanone;
MAPK, mitogen-activated protein kinase; CF, coronary flow; CHO-K1, Chinese
hamster ovary; HUVECs, human umbilical vein endothelial cells; HCASMCs, human
coronary artery smooth muscle cells; Fura-2 AM, Fura-2 pentaacetoxymethylester; SBP,
systemic blood pressure; HR, heart rate; MBP, mean blood pressure

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Abstract

Sphingosine 1-phosphate (S1P) induces diverse biological responses in various tissues by activating specific G protein-coupled receptors (S1P₁-S1P₅ receptors). The biological signaling regulated by S1P₃ receptor has not been fully elucidated because of the lack of a S1P₃ receptor-specific antagonist or agonist. We developed a novel S1P₃ receptor antagonist, 1-(4-chlorophenylhydrazono)-1-(4-chlorophenylamino)-3,3-dimethyl-2-butanone (TY-52156), and show here that the S1P-induced decrease in coronary flow (CF) is mediated by S1P₃ receptor. In functional studies, TY-52156 showed sub-micromolar potency and a high degree of selectivity for S1P₃ receptor. TY-52156, but not an S1P₁ receptor antagonist (VPC23019) or S1P₂ receptor antagonist (JTE013), inhibited the decrease in CF induced by S1P in isolated perfused rat hearts. We further investigated the effect of TY-52156 on both the S1P-induced increase in intracellular calcium ([Ca²⁺]_i) and Rho activation that are responsible for the contraction of human coronary artery smooth muscle cells. TY-52156 inhibited both the S1P-induced increase in [Ca²⁺]_i and Rho activation. In contrast, VPC23019 and JTE013 inhibited only the increase in [Ca²⁺]_i and Rho activation, respectively. We further confirmed that TY-52156 inhibited FTY-720-induced S1P₃ receptor-mediated bradycardia in vivo. These results clearly show that TY-52156 is both sensitive and useful as a S1P₃ receptor-specific antagonist, and reveal that S1P induces vasoconstriction by directly activating S1P₃ receptor and through a subsequent increase in [Ca²⁺]_i and Rho activation in vascular smooth muscle cells.

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Introduction

Sphingosine 1-phosphate (S1P) is a bioactive lysophospholipid mediator that is mainly released from activated platelets and induces many biological responses, including angiogenesis, vascular development and cardiovascular function (Siess, 2002; Takuwa et al., 2008; Yatomi, 2006). A wide variety of biological cellular responses to S1P have been ascribed to the presence of five S1P receptors, S1P₁-S1P₅ receptors, that belong to the family of G protein-coupled receptors (GPCRs). Furthermore, a variation of heterotrimeric G protein downstream of S1P receptors accounts for the diversity of cellular responses to S1P (Rosen et al., 2009). In addition to the coupling of S1P receptors and G proteins, the expression of the combination of S1P receptors determines multiple cellular responses. To identify the signaling that is specific for each receptor, S1P receptor antagonists have been developed and have contributed to our understanding of S1P-mediated signaling (Huwiler and Pfeilschifter, 2008).

S1P₁-S1P₅ receptors couple to different G proteins upon binding to S1P. While, S1P₁, S1P₄ and S1P₅ receptors mainly couple to Gi, S1P₂ and S1P₃ receptors couple to Gi, Gq and G_{12/13} (Rosen et al., 2009). The signal that converges from Gi-coupled S1P receptors inhibits the activation of adenylate cyclase and induces the activation of p44/p42 mitogen-activated protein kinase (MAPK). While S1P₁ receptor slightly increases intracellular calcium ([Ca²⁺]_i) through Gβγ, S1P₂ and S1P₃ receptors mainly increase [Ca²⁺]_i through the activation of phospholipase C (PLCβ) from Gq (Watterson et al., 2005). The deletion of S1P₃, but not S1P₂ receptor in mouse embryonic fibroblasts (MEFs) led to the marked inhibition of S1P-induced PLC activation, which suggests that S1P₃ receptor plays an important role in the S1P-induced increase in [Ca²⁺]_i (Ishii et al., 2002).

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S1P₂ and S1P₃ receptors also couple to G_{12/13} protein to activate a small GTPase, Rho, which is involved in the regulation of actin-cytoskeleton (Ryu et al., 2002; Sugimoto et al., 2003). Rho kinase is activated by Rho through the G_{12/13}-Rho guanine nucleotide exchange factor family. S1P-induced Rho activation has been shown to be significantly reduced in S1P₂, but not S1P₃, receptor-null MEFs (Ishii et al., 2002). Meanwhile, an association between S1P₃ receptor and Rho activation has been reported in cells expressing S1P₃ receptor (Sugimoto et al., 2003). An S1P-induced contraction of vascular smooth muscle cells has been ascribed to an increase in [Ca²⁺]_i and Rho activation (Ohmori et al., 2003; Watterson et al., 2005). S1P-induced vasoconstriction is significantly inhibited in cerebral arteries isolated from S1P₃ receptor-null mice, but not in those from S1P₂ receptor-null mice (Salomone et al., 2008). In addition, Y-27632, a selective Rho kinase inhibitor, inhibits S1P-induced vasoconstriction in canine cerebral arteries (Tosaka et al., 2001), indicating that S1P₃ receptor plays an indispensable role in S1P-induced vasoconstriction mediated by Rho-Rho kinase signaling. Although S1P decreases coronary flow (CF) in isolated perfused canine heart, the receptor subtype that is responsible for the S1P-induced reduction of CF has not yet been fully identified. To distinguish S1P₃ receptor-dependent signal from S1P₂ receptor-dependent signal, a S1P₃ receptor-specific antagonist has been needed.

We have developed an S1P₃ receptor antagonist, 1-(4-chlorophenylhydrazono)-1-(4-chlorophenylamino)-3,3-dimethyl-2-butanone (TY-52156). By confirming that TY-52156 has a selective antagonistic effect toward S1P₃ receptor, we can delineate the role of S1P₃ receptor-specific signaling in vascular contraction. Moreover, the effectiveness of TY-52156 in vivo was bolstered by evidence that S1P₃ receptor-dependent bradycardia was suppressed by the oral

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administration of TY-52156.

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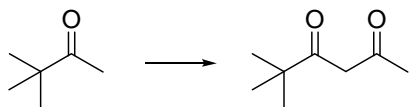
Materials and Methods

Materials

TY-52156 was synthesized in our laboratories. Materials were purchased from the following suppliers: S1P from BIOMOL International, L.P. (Plymouth Meeting, PA, USA); SEW2871, FTY-720 and FTY-720 (*S*)-Phosphate from Cayman Chemical (Ann Arbor, MI, USA); VPC23019 from Avanti Polar Lipids, Inc. (Alabaster, AL, USA); JTE013 from Tocris Bioscience (Southampton, UK); U46619 from Calbiochem (Darmstadt, Germany); HuMedia-EG2 and HuMedia-SG2 from Kurabo (Osaka, Japan); and membranes containing human S1P₁, S1P₂, S1P₃, or S1P₅ receptors from Millipore (Bedford, MA, USA).

Synthesis of TY-52156

5,5-Dimethyl-2,4-dihexanone (**1**)

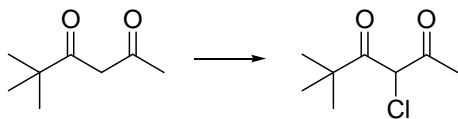


Diisopropyl ether (3 L) was placed in a 5 L 3-neck round-bottom flask and stirred mechanically. Potassium *tert*-butoxide (324 g, 2.25 mol, 1.5 eq) was suspended in the diisopropyl ether at 0 °C. Pinacolone (150 g, 1.50 mol, 1.0 eq) in ethyl acetate (440 mL, 4.50 mol, 3.0 eq) was slowly added dropwise so that the temperature would remain below 10 °C under ice-bath cooling. The reaction mixture was then stirred for 20 h at ambient temperature. Water (1 L) was added slowly so that the temperature would remain below 10 °C under ice-bath cooling. The separated organic layer was extracted with 1N sodium hydroxide (150 mL). The basic aqueous layer was carefully acidified with 6 N HCl (750 mL, 2.0 equivalents of base) at 0 °C and then extracted twice with

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petroleum ether (750 mL). The combined organic layer was washed with water (475 mL) and saturated brine (475 mL), dried over anhydrous sodium sulfate, filtered and evaporated at 200 mm Hg and ambient temperature. The remaining liquid was distilled under reduced pressure, with heating up to 80 °C. The title compound (**1**) was obtained as a colorless oil (126 g, 0.89 mol, 59%); ¹H-NMR (300 MHz, CDCl₃) δ: 1.17 (s, 9 H), 2.08 (s, 3 H), 5.61 (s, 1 H); bp.: 62-69 °C (20 mmHg).

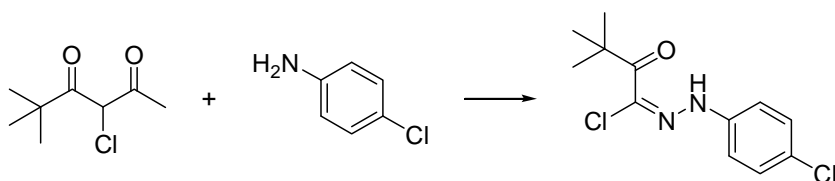
3-Chloro-5,5-dimethyl-2,4-dihexanone (**2**)



5,5-Dimethyl-2,4-dihexanone (**1**) (35.4 g, 249 mmol, 1.0 eq) was dissolved in chloroform (700 mL) and stirred mechanically. Sulfuryl chloride (260 mL, 324 mmol, 1.3 eq) in chloroform (130 mL) was slowly added dropwise so that the temperature would remain below 5 °C under ice-bath cooling. The reaction mixture was then stirred for 2 h at 25 °C, and then quenched with water (500 mL) at 0 °C. The separated organic layer was washed three times with water (500 mL), dried over anhydrous sodium sulfate, filtered and evaporated. The residue was purified by distillation under reduced pressure, with heating to 70 °C. The title compound (**2**) was obtained as a yellow oil (41.5 g, 235 mmol, 94%); ¹H-NMR (300 MHz, CDCl₃) δ: 1.23 (s, 9 H), 2.38 (s, 3 H), 5.09 (s, 1 H); bp.: 67-69 °C (1.5 mmHg).

[1-Chloro-1-(4-chlorophenylhydrazono)]-3,3-dimethyl-2-butanone (**3**)

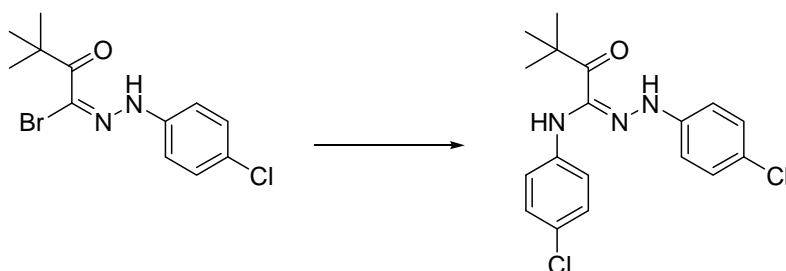
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4-Chloroaniline (29 g, 226 mmol, 1.0 eq) was added to 6 N hydrochloric acid (158 mL, 951 mmol, 4.2 eq) and water (68 mL) and stirred for 15 min at 0 °C. Sodium nitrite (17 g, 249 mmol, 1.1 eq) in water (90 mL) was slowly added dropwise so that the temperature would remain below 5 °C under ice-bath cooling. The reaction mixture was stirred for 1 h at 0 °C to prepare a solution of diazonium salt. 3-Chloro-5,5-dimethyl-2,4-hexanone (**2**) (40 g, 226 mmol, 1.0 eq) was dissolved in pyridine (158 mL) and water (158 mL) at 0 °C. The previously prepared diazonium salt solution was slowly added dropwise so that the temperature would remain below 10 °C, and the resulting mixture was then vigorously stirred for 2 h, with warming from 0 to 25 °C. The reaction mixture was extracted with ethyl acetate (452 mL), washed twice with 2N HCl (1 L) and saturated brine (452 mL), dried over anhydrous sodium sulfate, filtered and evaporated. The resulting crude product was diluted with methanol (226 mL, 1M solution), refluxed for 1 h and then cooled to 0 °C. The precipitated crude crystals were collected by filtration, washed with petroleum ether, and dried under reduced pressure to give the title compound (**3**) as a yellow solid (24 g, 89 mmol, 39%); ¹H-NMR (300 MHz, CDCl₃) δ: 1.43 (s, 9H), 7.12 (d, *J* = 8.7 Hz, 2H), 7.33 (d, *J* = 8.7 Hz, 2H), 8.34 (s, 1H); m.p.: 129-131 °C.

2-(4-Chlorophenylhydrazono)-2-(4-chlorophenylamino)acetophenone (**TY-52156**)

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[1-Chloro-1-(4-chlorophenylhydrazono)]-3,3-dimethyl-2-butanone (**3**) (22.4 g, 82.1 mmol, 1.0 eq) and 4-chloroaniline (11.5 g, 90.4 mmol, 1.1 eq) were dissolved in ethanol (274 mL), and triethylamine (13.7 mL, 98.6 mmol, 1.2 eq) was then added at 0 °C. The resulting mixture was stirred for 3 h at ambient temperature. The reaction mixture was evaporated, quenched with water (82 mL), and diluted with ethyl acetate (165 mL). The organic layer was washed with water (165 mL) and saturated brine (165 mL), dried over anhydrous sodium sulfate, filtered and evaporated. The resulting crude crystals were washed with a solvent mixture of hexane-ethyl acetate (20:1) and dried under reduced pressure to obtain the title compound (**TY-52156**) as a yellow powder (24.8 g, 68.1 mmol, 83%); ¹H-NMR (300 MHz, CDCl₃) δ: 1.48 (s, 9H), 6.55 (d, *J* = 8.4 Hz, 2H), 6.76 (s, 1H), 6.98 (d, *J* = 8.8 Hz, 2H), 7.19-7.26 (m, 5H); MS (ESI) *m/z* = 362 (M-H)⁺; mp.: 90-91 °C.

Cell Culture

Chinese hamster ovary (CHO-K1) cells that stably expressed human S1P₁ (S1P₁-CHO), S1P₂ (S1P₂-CHO) or S1P₃ (S1P₃-CHO) receptors were maintained as described previously (Koide et al., 2007). A human recombinant S1P₄ receptor-expressing cell line (S1P₄-Chem) was purchased from Millipore. Human umbilical vein endothelial cells (HUVECs) purchased from DS Pharma Biomedical (Osaka, Japan) were cultured on collagen-coated dishes in HuMedia-EG2. Human coronary artery smooth muscle

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cells (HCASMCs) purchased from Kurabo were cultured in HuMedia-SG2.

Measurement of the Intracellular Calcium Concentration

[Ca²⁺]_i in S1P₁-, S1P₂-, S1P₃-CHO, and S1P₄-Chem was measured using the calcium-sensitive dye Fura-2 AM, as described previously (Koide et al., 2007). The fluorescence (excitation at 340 and 380 nm; emission at 510 nm) was measured with a FLEXStation II (Molecular Devices). The ratio of the fluorescence intensity at two wavelengths (FR340/380) was calculated. The K_i value for TY-52156 was estimated from Ca²⁺ responses as described previously (Ohta et al., 2003).

[³H]-S1P Binding Assay

A [³H]-S1P binding assay was performed as described by Lim et al. (2003) with minor modifications. The cell membrane (60 µg/mL) was incubated with binding buffer containing [³H]-S1P (1 nM, about 40,000 dpm per well) and vehicle or each concentration of TY-52156 (µM) for 30 min at 25°C. Radioactivity was measured by a liquid scintillation counter after the addition of scintillation cocktail solution. Nonspecific binding was defined as the amount of radioactivity bound to the cells in the presence of unlabeled S1P (3.0 µM). Specific binding was calculated by subtracting nonspecific binding from total binding.

GTP-binding Assay

Europium-GTP (Eu-GTP) binding was determined using a DELFIA GTP Binding Assay Kit (Perkin-Elmer Life Sciences, Wallac, Turku, Finland). Samples were incubated in AcroWell filter plates (PALL, Ann Arbor, MI, USA) for 60 min (S1P₁ and S1P₅) or 90

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min (S1P₂ and S1P₃) at 30°C. The reaction was started by adding membranes (48 µg/mL) containing human S1P₁, S1P₂, S1P₃ or S1P₅ receptors to the assay buffer (20 mM HEPES, pH7.4, 5 mM MgCl₂, 100 mM NaCl, 1.2 mg/mL Saponin, 10 µM GDP and 10 nM Eu-GTP at 30°C) including S1P (0.1 µM) and vehicle or the desired concentration (µM) of the test drug (TY-52156 or VPC23019). The reaction was terminated by rapid filtration and the filter was washed five times with 200 µL of ice-cold washing solution in a vacuum manifold. The plate was measured by time-resolved fluorescence (340 nm excitation/615 nm emission) using a EnVision (Perkin-Elmer Life Sciences).

Western Blot Analysis for p44/p42 MAPK

S1P₁-, S1P₂- and S1P₃-CHO (2.0×10⁵ cells) were plated on 6-well plates and cultured with Nutrient Mixture F-12 Ham (Sigma) containing 1% FBS for 4 h before the experiments. The cells were treated with vehicle, TY-52156 (TY) (10 µM), VPC23019 (VPC) (10 µM) or JTE013 (JTE) (1.0 µM) for 10 min and then with vehicle or S1P (0.1 µM) for 5 min at 37°C. The cells were lysed in CellLyticM containing Protease Inhibitor Cocktails and Phosphatase Inhibitor Cocktails (Sigma) for 10 min at 4°C. The lysate was centrifuged at 13,000×g for 15 min at 4°C and supernatant was transferred to a fresh tube. The protein concentration was determined using the Bradford method. Equal amounts of proteins were resuspended in 4×Sample Buffer (Wako Pure Chemical Industries), boiled for 5 min and separated by 10% SDS-PAGE. After being transferred to a polyvinylidene fluoride (PVDF) membrane, the membranes were blocked in Block Ace (DS Pharma Biomedical) and immunoblotted with antibodies of phospho-p44/p42 MAPK or p44/p42 MAPK (1:1000, Cell Signaling

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Technology). The signals were visualized by an Amplified Alkaline Phosphatase Goat Anti-Rabbit Immun-Blot Assay Kit (Bio-Rad) according to the manufacturer's instructions. Quantitative analyses of immunoblots were performed using Quantity One version 4.2.2 software (Bio-Rad). The relative percentage compared with the vehicle was calculated and expressed as the mean \pm S.E.M.

Measurement of Coronary Flow

All animal experiments were reviewed and approved by the Experimental Animal Committee in our laboratories. Male Sprague-Dawley (SD) rats (300-350 g; Nihon SLC) were anesthetized by the injection of pentobarbital (50 mg/kg i.p.). After thoracotomy, their hearts were rapidly excised and perfused at 37°C in a Langendorff manner with Krebs-Henseleit bicarbonate buffer (constant perfusion pressure of 70 \pm 5 mmHg) of the following composition (in mM): NaCl 118; KCl 4.7; KH₂PO₄ 1.2; MgSO₄ 1.2; CaCl₂ 2.5; NaHCO₃ 24.9; EDTA/2Na 0.027; ascorbic acid 0.057 and glucose 11.1, pH 7.4 at 37°C, bubbled with 95% O₂ and 5% CO₂ (pO₂>550 mmHg). A modified water-filled latex balloon (LB-2, Technical Service Corporation) was inserted into the left ventricle via the left atrium with a pressure transducer (DX-360, Ohmeda) connected to an amplifier (AP-601G, Nihon Kohden). Left ventricular end-diastolic pressure was adjusted to about 5-10 mmHg. To measure coronary flow (CF), a Cannulating-type Flow Probe (FF-030T, Nihon Kohden) connected to an electro-magnetic blood-flow meter (MFV-3700, Nihon Kohden) was inserted to the perfusion line that was connected to the heart. After a 15-min period for equilibration, vehicle, TY-52156 (TY) (0.1 μ M), VPC23019 (VPC) (0.1 μ M) or JTE013 (JTE) (0.1 μ M) was infused for 10 min by an infusion pump (Harvard Apparatus) through a

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drug-infusion line connected to the main perfusion line at a flow rate of 1/100 the CF rate. After drug treatment, vehicle or the indicated concentration of S1P (μM) or U46619 (0.1 μM) was added to the same line. CF was measured before and 10 min after the infusion of S1P or U46619. The relative percentage compared to the vehicle was calculated from the CF rate.

Contractile Response in Cerebral Arteries

Beagle dogs (Oriental Yeast) were anesthetized by the injection of pentobarbital (30 mg/kg i.p.). The cerebral arteries were rapidly excised and mounted in organ chambers containing Krebs buffer of the following composition (in mM): NaCl 118.0, KCl 4.7, CaCl_2 2.5, KH_2PO_4 1.2, MgSO_4 1.2, NaHCO_3 25.0 and glucose 11.0, pH 7.2 at 37°C, bubbled with 95% O_2 and 5% CO_2 . After equilibration, cerebral arteries were exposed to 60 mM KCl until the contractile responses were stabilized. After washout and recovery, contractile responses to S1P were measured every 10 min after the addition of the indicated concentration of S1P (μM). In another experiment, the cerebral arteries were contracted by S1P (5.0 μM), and then an increasing amount of vehicle or TY-52156 (up to 10 μM) was applied to the organ chambers. Relaxation responses were measured every 10 min after addition of the indicated concentration of vehicle or TY-52156 (μM). The degree of contraction compared to vehicle was calculated with PRISM version 4 statistical software (GraphPad) and expressed as the percent contraction compared to that induced by S1P.

Immunoprecipitation and Western Blot

HCASMCs were seeded at 5×10^5 cells in a culture dish. After they reached

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confluence, cells were pretreated with vehicle or the indicated concentration (μM) of test drug for 10 min, and then with vehicle or S1P (0.1 μM) for 3 min at 37°C. The amounts of activated Rho (GTP-Rho) were measured by a Rho Activation Assay Biochem Kit (Cytoskeleton) according to the manufacturer's instructions. Quantitative analysis was performed as described above. The density ratio of GTP-Rho to total Rho was measured and its vehicle value was set to 1.0.

Pharmacokinetic Analysis

Male SD rats were purchased from Nihon SLC. Blood samples were collected from the jugular vein at 1, 2, 4, 6, 8 and 24 h after the start of the administration of TY-52156-HCl. Samples were placed into sodium heparinized tubes and subjected to centrifugation at 14,000 \times g for 10 min at 4°C to separate the plasma. Plasma concentrations were quantified by an API 4000 (TM) LC/MS/MS System (Applied Biosystems/MDS SCIEX, Ontario, Canada). The mean peak plasma concentration (C_{max}) and time to reach C_{max} (T_{max}) were estimated from actual measurements. The half-life ($T_{1/2}$) was calculated with WinNonlin ver. 2.1 software (Pharsight, Mountain View, CA).

Measurement of Systemic Blood Pressure, Heart Rate and Mean Blood Pressure

Male SD rats (290-340 g) were purchased from Nihon SLC. Systemic blood pressure (SBP) and heart rate (HR) were measured in the conscious state with a tail-cuff blood pressure analyzer (MK2000, Muromachi-kikai). In another experiment, SD rats were anesthetized by the injection of pentobarbital (50 mg/kg i.p.) and cannulas were placed in a carotid artery and femoral vein. Mean blood pressure (MBP) was measured with a

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pressure transducer (DX-360) that was connected to the cannula placed in a carotid artery. HR was measured with a tachometer (1321; NEC-San-ei). MBP and HR were measured before, and 10 and 20 min after the injection of FTY-720 (1.0 mg/kg i.v.).

Statistical Analysis

Experimental values are expressed as mean±S.E.M. The Student *t*-test or ANOVA followed by Dunnett's multiple-comparison test was used to statistically analyze differences between groups. $P < 0.05$ was considered to be significant.

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Results

TY-52156 is a Potent S1P₃ Receptor Antagonist

S1P₃ receptor contributes to the S1P-induced increase in $[Ca^{2+}]_i$ (Ishii et al., 2002). To identify potent S1P₃ receptor antagonists, first, we screened a diverse compound collection (7,500 compounds) by a Ca^{2+} fluorescent assay using S1P₃-CHO (Supplementary Methods). The hit criterion was defined as more than 50% inhibition of the S1P (0.01 μ M)-induced increase in $[Ca^{2+}]_i$ at 10 μ M. Second, several possible compounds were pruned to confirm the dose-dependent and specific inhibition of S1P₃ receptor. Third, we synthesized derivatives of the active compounds to improve their potency and selectivity toward S1P₃ receptor. As a result, we identified TY-52156 as a potent S1P₃ receptor antagonist (Fig. 1A). TY-52156 preferentially inhibited the S1P-induced increase in $[Ca^{2+}]_i$ in S1P₃-CHO, rather than S1P₁-, S1P₂-CHO and S1P₄-Chem (Fig. 1B and Supplementary Fig. 1 (as the direct ratio of fluorescence)). The dose-dependent $[Ca^{2+}]_i$ increase elicited by S1P in S1P₃-CHO was inhibited by TY-52156 in a competitive manner (Fig. 1C), and the K_i value was estimated to be 110 nM for S1P₃ receptor.

In addition to S1P receptor-expressing cell lines, we used HUVECs to confirm the specificity of TY-52156 for endogenous S1P₃ receptor. S1P₁ and S1P₃ receptors but not other S1P receptors are expressed on HUVECs and induce $[Ca^{2+}]_i$ elevation (Sensken et al., 2008), which is consistent with the finding that TY-52156 and VPC23019 inhibited the S1P-induced increase in $[Ca^{2+}]_i$ in HUVECs (Supplementary Fig. 2). Furthermore, the combination of TY-52156 and VPC23019 showed greater inhibitory activity than either compound alone (Supplementary Fig. 2). Thus, S1P induces $[Ca^{2+}]_i$ elevation via S1P₁ and S1P₃ receptors in HUVECs. To assess the

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inhibitory effect of the TY-52156 on S1P-induced increase in $[Ca^{2+}]_i$ through S1P₃, but not S1P₁ receptor, the SEW2871 (a S1P₁ receptor-specific agonist)-induced increase in $[Ca^{2+}]_i$ in HUVECs was evaluated with or without TY-52156. Pretreatment with VPC23019 (a S1P₁ receptor antagonist) significantly inhibited the SEW2871-induced increase in $[Ca^{2+}]_i$ through S1P₁ receptor (Fig. 1D). In contrast, this inhibition was blunted by pretreatment with TY-52156. These results suggest that TY-52156 inhibits the S1P₃ receptor-dependent increase in $[Ca^{2+}]_i$.

TY-52156 is a Selective S1P₃ Receptor Antagonist

Using a [³H]-S1P binding assay, we found that TY-52156 inhibited the specific binding of [³H]-S1P to the membrane fraction of S1P₃-CHO in a dose-dependent manner (Fig. 2A). The total [³H]-S1P binding and nonspecific binding were 2156 ± 315 and 883 ± 109 , respectively (mean dpm values \pm S.E.M., n=5). To further characterize the antagonist actions of TY-52156, we performed Eu-GTP binding to membranes prepared from cells expressing the human S1P₁, S1P₂, S1P₃ or S1P₅ receptors. The Eu-GTP binding assay has become a powerful alternative to the [³⁵S]GTP γ S binding assay (Moreland et al., 2004). TY-52156 showed sub-micromolar potency and a high degree of selectivity for S1P₃ receptor (Fig. 2B).

We examined the selective inhibitory effect of TY-52156 on S1P-induced p44/p42 MAPK phosphorylation in S1P₁-, S1P₂- and S1P₃-CHO. TY-52156, VPC23019 and JTE013 inhibited S1P-induced p44/p42 MAPK phosphorylation only in S1P₃-, S1P₁- and S1P₂-CHO, respectively (Fig. 2C and 2D). While VPC23019 is at least one-fifth less active toward S1P₃ receptor than S1P₁ receptor (Davis et al., 2005), it did not have an inhibitory effect on S1P-induced p44/p42 MAPK phosphorylation in S1P₃-CHO

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under our experimental conditions.

We further confirmed the selectivity of TY-52156 (10 μ M) by examining its inhibitory effects on 24 GPCRs and three ion channels (all % inhibitions < 30%, see Supplemental Table). These results indicate that TY-52156 is a potent S1P₃ receptor-selective antagonist.

S1P Reduces Coronary Flow via S1P₃ Receptor

S1P is released from activated platelets and thus is thought to be involved in thrombosis-related vascular diseases such as acute coronary syndrome (Siess, 2002). Recent studies have reported that intravenous injection of S1P decreases myocardial perfusion via S1P₃ receptor in vivo (Levkau et al., 2004). To investigate whether TY-52156 regulates CF, we examined its effect on S1P-dependent CF regulation. Consistent with previous reports (Sugiyama et al., 2000), we found that S1P dose-dependently decreased CF in perfused rat heart (Fig. 3A). Hearts were perfused with a solution containing each S1P receptor antagonist prior to S1P treatment. TY-52156, but not VPC23019 or JTE013, significantly restored the S1P-dependent reduction of CF (Fig. 3B). Meanwhile, TY-52156 did not affect the reduction of CF caused by a stable analogue of thromboxane A₂ (TXA₂), U46619. These results indicate that S1P reduces CF via S1P₃ receptor.

S1P Induces the Vasoconstriction of Canine Cerebral Arteries via S1P₃ Receptor

To investigate whether S1P₃ receptor expressed in the vasculature plays a role in S1P-induced vasoconstriction, we focused on the effect of TY-52156 on S1P-induced vasoconstriction in isolated canine cerebral arteries. S1P dose-dependently induced

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vasoconstriction (Fig. 4A), which is consistent with previous reports (Tosaka et al., 2001). TY-52156 cumulatively induced the relaxation of canine cerebral arteries that had been precontracted by S1P (Fig. 4B). These results suggest that S1P induces the vasoconstriction of isolated canine cerebral arteries via S1P₃ receptor.

S1P Induces both Rho Activation and the Increase in Ca²⁺ via S1P₃ Receptor in HCASMCs

Vascular tone balances relaxation and constriction in smooth muscle cells (Watterson et al., 2005). Vasorelaxation is mainly mediated by nitric oxide released from endothelial cells, where S1P activates endothelial nitric oxide synthase (eNOS). Vasoconstriction is presumably regulated by S1P-induced Rho activation and the increase in [Ca²⁺]_i in vascular smooth muscle cells. Since arteries were contracted by S1P stimulation, we assumed that S1P-mediated contraction dominated S1P-mediated relaxation in isolated perfused heart and arteries. Therefore, we hypothesized that the S1P-induced decrease in CF in isolated perfused rat heart might be ascribed to the contraction of coronary artery smooth muscle cells expressing S1P₃ receptor. We tested whether TY-52156 inhibited S1P-induced Rho activation and the increase in [Ca²⁺]_i in HCASMCs.

S1P-induced Rho activation in HCASMCs was inhibited by TY-52156 (Fig. 5A). JTE013 also inhibited S1P-induced Rho activation (Fig. 5B), which is consistent with previous studies (Arikawa et al., 2003). The combination of TY-52156 and JTE013 showed greater inhibitory activity than either compound alone (Fig. 5C). In contrast, VPC23019 did not inhibit S1P-induced Rho activation. SEW2871 did not induce Rho activation (Fig. 5D). Pretreatment with TY-52156 prevented the S1P-induced increase in [Ca²⁺]_i in HCASMCs (Fig. 6). Meanwhile, pretreatment with VPC23019, but not

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JTE013, attenuated the S1P-induced increase in $[Ca^{2+}]_i$ in HCASMCs. Collectively, these results suggest that S1P₃ receptor is responsible for both S1P-induced Rho activation and the increase in $[Ca^{2+}]_i$, whereas S1P₁ and S1P₂ receptors are involved in the increase in $[Ca^{2+}]_i$ and Rho activation, respectively.

TY-52156 Suppresses S1P₃ Receptor-induced Bradycardia In Vivo

Finally, we sought to confirm the effects of TY-52156 in living animals. To test whether TY-52156 inhibits S1P₃ receptor in vivo, we examined the antagonistic effects of TY-52156 on the FTY-720 (nonselective S1P receptor agonist)-induced transient reduction of HR.

The oral bioavailability of TY-52156 in SD rats was estimated to be 70.9 % (Fig. 7A). To determine the pretreatment time prior to FTY-720 injection, the plasma concentrations of TY-52156 were measured (Fig. 7B). We also confirmed that oral administration (p.o.) of TY-52156 did not affect either HR or SBP in conscious rats (Fig. 7C and 7D).

Although FTY-720 is a broad agonist of S1P receptors, it induces bradycardia, which has been shown to be mediated by the activation of S1P₃ receptor using S1P₃ receptor-null mice (Forrest et al., 2004; Sanna et al., 2004). FTY-720 (i.v.)-induced sinus bradycardia was observed from 10 to 20 min (Fig. 7E). Pretreatment with TY-52156 (p.o.) partially but significantly attenuated FTY-720-induced bradycardia, but did not affect the FTY-720-induced elevation of MBP in unconscious rats (Fig. 7E and 7F).

To complement the observation that TY-52156 inhibited FTY-720-induced bradycardia in vivo, dose-dependent inhibition of the FTY-720-induced cellular

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response was clarified. FTY-720 is phosphorylated to the active metabolite FTY-720 phosphate (FTY-720-P) in vivo (Zemann et al., 2006). We examined the inhibitory effect of TY-52156 on the FTY-720-P-induced increase in $[Ca^{2+}]_i$ in S1P₃-CHO. Pretreatment with TY-52156 prevented the FTY-720-P-induced increase in $[Ca^{2+}]_i$ in a dose-dependent manner (Fig. 8). Collectively, these results indicate that the oral administration of TY-52156 inhibits S1P₃ receptor-dependent bradycardia in vivo.

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Discussion

TY-52156 was identified as a potent and selective antagonist of S1P₃ receptor. Based on its ability to inhibit Ca²⁺ responses and the results of a Eu-GTP binding assay, TY-52156 was about 10 to 30 times more potent for S1P₃ receptor than for S1P₁, S1P₂, S1P₄ or S1P₅ receptor, and the K_i value for S1P₃ receptor was estimated to be 110 nM. TY-52156 caused a parallel rightward shift of the dose-response curve for the S1P-induced increase in [Ca²⁺]_i, which suggested competitive antagonism for S1P₃ receptor. Furthermore, TY-52156 did not have any significant effects on 24 GPCRs or three ion channels. Therefore, TY-52156 is a useful tool for studying S1P₃ receptor signaling.

VPC23019 has been described as an S1P₁ and S1P₃ receptor antagonist, with pK_b values (-logM) of 7.49 ± 0.15 and 5.98 ± 0.08 for the S1P₁ and S1P₃ receptors, respectively (Davis et al., 2005). Although VPC23019 (10 μM) did not inhibit p44/p42 MAPK phosphorylation in S1P₃-CHO, dose-dependent inhibition was observed at higher concentrations (30 to 100 μM) (Supplementary Fig. 3A). On the other hand, VPC23019 dose-dependently inhibited Eu-GTP binding to membranes (S1P₃) at from 1 to 10 μM (Supplementary Fig. 3B), which is similar to previous findings (Davis et al., 2005). One possible explanation is that VPC23019 did not inhibit the cellular response on S1P₃-CHO because of the difference in sensitivity between membrane-based and whole cell-based assay conditions. Additionally, since VPC23019 has been characterized in T24 cells (human bladder carcinoma) that stably expressed S1P₃ receptor (Davis et al., 2005), host-cell-specific differences between T24 cells and our CHO-K1 cells might play a role.

We demonstrated that S1P₃ receptor is central to S1P-regulated CF. CF is

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increased or decreased physiologically in response to the oxygen demand of the heart muscle. Thrombosis and atherosclerosis decrease CF by releasing S1P, TXA₂, and platelet-derived growth factor from activated platelets and by narrowing the lumen of the coronary arteries, respectively (Heldin and Westermark, 1999; Pomposiello et al., 1997). Although S1P has been reported to decrease CF, it is not well known how S1P induces vasoconstriction. The deletion of S1P₃ receptor in mice led to inhibition of the S1P-induced decrease in myocardial perfusion (Levkau et al., 2004). We found that TY-52156, but not VPC23019 or JTE013, attenuated the S1P-dependent reduction of CF. Therefore, S1P₃ receptor is responsible for the S1P-induced decrease in cardiac coronary flow. Since S1P₃ receptor is highly expressed in the smooth muscle of small coronary vessels (Himmel et al., 2000; Mazurais et al., 2002), reduction of CF by S1P may primarily depend on the vasoconstriction of microvascular smooth muscle cells.

We focused on the mechanism by which S1P₃ receptor regulates the contraction of smooth muscle cells. There are two main signals that induce actomyosin-based contraction: an increase in [Ca²⁺]_i and Rho activation (Watterson et al., 2005). We observed that TY-52156 inhibited the S1P-induced increase in [Ca²⁺]_i and Rho activation in HCASMCs. In clear contrast, VPC23019 and JTE013 only inhibited the increase in [Ca²⁺]_i and Rho activation, respectively. Thus, S1P₃ receptor-mediated signal through both the increase in [Ca²⁺]_i and Rho activation, which lead to vasoconstriction, can account for our finding that TY-52156, but not VPC23019 or JTE013, preserved the S1P-dependent reduction of CF in perfused rat heart. Although Rho kinase inhibition has been believed to cause vasorelaxation (Tosaka et al., 2001), it is unclear why JTE013 did not alter the S1P-dependent reduction of CF. One possible explanation is the difference in the expression of S1P receptor subtypes in the

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vasculature (Coussin et al., 2002; Mazurais et al., 2002).

Sustained vascular spasm after subarachnoid hemorrhage and cerebral infarction has been shown to result in the extension of brain damage (Tosaka et al., 2001). The involvement of S1P₃ receptor in vasospasm has been reported using S1P₃ receptor-null mice (Salomone et al., 2008). The intracarotid injection of S1P decreases cerebral blood flow in vivo (Salomone et al., 2003). Thus, TY-52156 may potentially inhibit the S1P-induced vasospasm of cerebral arteries, since we found that TY-52156 attenuated S1P-induced vascular contraction in canine cerebral arteries.

S1P has opposite effects on vasculature: vasorelaxation and vasoconstriction. S1P₁ and S1P₃ receptors have been linked to the activation of NO synthesis in vascular endothelial cells (Dantas et al., 2002; Igarashi and Michel, 2000; Nofer et al., 2004). However, we confirmed that activation of S1P₃ receptor led to vasoconstriction in smooth muscle cells. Therefore, the net effect of S1P₃ receptor on vasorelaxation and vasoconstriction depends on the function of vascular endothelial cells or the expression profile of S1P receptor subtypes in the vasculature (Fig. 9). Endothelial-dependent vasorelaxation is supported by the fact that various vasoconstrictors, including acetylcholine and ergonovine, cause endothelium-dependent vasorelaxation via a NO-dependent mechanism in healthy subjects (Davignon and Ganz, 2004; Kawano and Ogawa, 2004; Kugiyama et al., 1996). This vasorelaxation is impaired in patients with endothelial dysfunction. Thus, S1P at least contributes to pathological processes that involve endothelial dysfunction, such as vasospasm and myocardial infarction.

TY-52156 might become a potent probe for assessing S1P₃ receptor-dependent signal in vivo. FTY-720 binds to all S1P receptors except S1P₂ receptor (Huwiler and Pfeilschifter, 2008). While FTY-720-induced bradycardia is mainly caused by the

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S1P₃ receptor-mediated activation of cardiac G protein-gated potassium channel (I_{K_{ACh}}), it has been reported to be associated with I_{K_{ACh}}-independent mechanisms through FTY-720 induction (Himmel et al., 2000; Forrest et al., 2004; Koyrakh et al., 2005). Based on the study of I_{K_{ACh}}-deficient mice, other pacing-related currents such as the hyperpolarization-activated inward current (I_f) and the voltage-gated calcium current (I_{Ca,L}) may be involved in the bradycardia with FTY-720 (Koyrakh et al., 2005). In addition, despite the lack of S1P₃ receptor agonism, a recent clinical study has reported that a selective S1P₁/S1P₅ receptor agonist (BAF-312) decreased the heart rate in healthy subjects (Gergely et al., 2009). Our result showed that FTY-720 (i.v.) decreased HR and elevated MBP in rats. Pretreatment with TY-52156 prior to FTY-720 partially restored the FTY-720-induced HR reduction, but did not attenuate the elevation of MBP. Since the oral administration of TY-52156 alone did not affect HR or SBP in conscious rats, this result probably means that there is no effect on I_f current to modulate HR. We also found that TY-52156 did not affect I_{Ca,L} in guinea-pig ventricular myocytes (Supplemental Table). Thus, these results indicated that FTY-720-induced bradycardia may be involved in the mechanism, except through S1P₃ receptor. While FTY-720 induced an elevation of MBP in a clinical study, the mechanism was not clear (Kappos et al., 2006). Therefore, TY-52156 inhibits S1P₃ receptor-dependent HR reduction in vivo and thus is a potent probe for elucidating the role of S1P₃ receptor in animal models.

In conclusion, TY-52156 is a potent and selective antagonist of S1P₃ receptor. This compound may help us to distinguish S1P₃ receptor-dependent signals from S1P₁ and S1P₂ receptor-dependent signals in vitro and in vivo. S1P₃ receptor is responsible for the S1P-induced decrease in CF, and an S1P₃ receptor antagonist may be useful for

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the treatment of S1P-induced vascular diseases including vasospasm.

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References

- Arikawa K, Takuwa N, Yamaguchi H, Sugimoto N, Kitayama J, Nagawa H, Takehara K and Takuwa Y (2003) Ligand-dependent inhibition of B16 melanoma cell migration and invasion via endogenous S1P2 G protein-coupled receptor. Requirement of inhibition of cellular RAC activity. *J Biol Chem* **278**(35):32841-32851.
- Coussin F, Scott RH, Wise A and Nixon GF (2002) Comparison of sphingosine 1-phosphate-induced intracellular signaling pathways in vascular smooth muscles: differential role in vasoconstriction. *Circ Res* **91**(2):151-157.
- Dantas AP, Igarashi J and Michel T (2002) Sphingosine 1-phosphate and control of vascular tone. *Am J Physiol Heart Circ Physiol* **284**(6):H2045-2052.
- Davignon J and Ganz P (2004) Role of endothelial dysfunction in atherosclerosis. *Circulation* **109**(23 Suppl 1):III27-32.
- Davis MD, Clemens JJ, Macdonald TL and Lynch KR (2005) Sphingosine 1-phosphate analogs as receptor antagonists. *J Biol Chem* **280**(11):9833-9841.
- Forrest M, Sun SY, Hajdu R, Bergstrom J, Card D, Doherty G, Hale J, Keohane C, Meyers C, Milligan J, Mills S, Nomura N, Rosen H, Rosenbach M, Shei GJ, Singer II, Tian M, West S, White V, Xie J, Proia RL and Mandala S (2004) Immune cell regulation and cardiovascular effects of sphingosine 1-phosphate receptor agonists in rodents are mediated via distinct receptor subtypes. *J Pharmacol Exp Ther* **309**(2):758-768.
- Gergely P, Wallström E, Nuesslein-Hildesheim B, Bruns C, Zécéri F, Cooke N, Traebert M, Tuntland T, Rosenberg M and Saltzman M (2009) Phase I study with the selective S1P1/S1P5 receptor modulator BAF312 indicates that S1P1 rather than

MOL#61481

S1P3 mediates transient heart rate reduction in humans. *Multiple Sclerosis* 15(9 Suppl):S235-S236.

Heldin CH and Westermark B (1999) Mechanism of action and in vivo role of platelet-derived growth factor. *Physiol Rev* **79**(4):1283-1316.

Himmel HM, Meyer Zu Heringdorf D, Graf E, Dobrev D, Kortner A, Schuler S, Jakobs KH and Ravens U (2000) Evidence for Edg-3 receptor-mediated activation of I(K.ACh) by sphingosine-1-phosphate in human atrial cardiomyocytes. *Mol Pharmacol* **58**(2):449-454.

Huwiler A and Pfeilschifter J (2008) New players on the center stage: sphingosine 1-phosphate and its receptors as drug targets. *Biochem Pharmacol* **75**(10):1893-1900.

Igarashi J and Michel T (2000) Agonist-modulated targeting of the EDG-1 receptor to plasmalemmal caveolae. eNOS activation by sphingosine 1-phosphate and the role of caveolin-1 in sphingolipid signal transduction. *J Biol Chem* **275**(41):32363-32370.

Ishii I, Ye X, Friedman B, Kawamura S, Contos JJ, Kingsbury MA, Yang AH, Zhang G, Brown JH and Chun J (2002) Marked perinatal lethality and cellular signaling deficits in mice null for the two sphingosine 1-phosphate (S1P) receptors, S1P(2)/LP(B2)/EDG-5 and S1P(3)/LP(B3)/EDG-3. *J Biol Chem* **277**(28):25152-25159.

Kappos L, Antel J, Comi G, Montalban X, O'Connor P, Polman CH, Haas T, Korn AA, Karlsson G and Radue EW (2006) Oral fingolimod (FTY720) for relapsing multiple sclerosis. *N Engl J Med* **355**(11):1124-1140.

Kawano H and Ogawa H (2004) Endothelial dysfunction and coronary artery spasm.

MOL#61481

Curr Drug Targets Cardiovasc Haematol Disord **4**(1):23-33.

Koide Y, Uemoto K, Hasegawa T, Sada T, Murakami A, Takasugi H, Sakurai A, Mochizuki N, Takahashi A and Nishida A (2007) Pharmacophore-based design of sphingosine 1-phosphate-3 receptor antagonists that include a 3,4-dialkoxybenzophenone scaffold. *J Med Chem* **50**(3):442-454.

Koyrakh L, Roman MI, Brinkmann V and Wickman K (2005) The heart rate decrease caused by acute FTY720 administration is mediated by the G protein-gated potassium channel I. *Am J Transplant* **5**(3):529-536.

Kugiyama K, Yasue H, Okumura K, Ogawa H, Fujimoto K, Nakao K, Yoshimura M, Motoyama T, Inobe Y and Kawano H (1996) Nitric oxide activity is deficient in spasm arteries of patients with coronary spastic angina. *Circulation* **94**(3):266-271.

Levkau B, Hermann S, Theilmeyer G, van der Giet M, Chun J, Schober O and Schafers M (2004) High-density lipoprotein stimulates myocardial perfusion in vivo. *Circulation* **110**(21):3355-3359.

Lim HS, Oh YS, Suh PG and Chung SK (2003) Syntheses of sphingosine-1-phosphate stereoisomers and analogues and their interaction with EDG receptors. *Bioorg Med Chem Lett* **13**(2):237-240.

Mazurais D, Robert P, Gout B, Berrebi-Bertrand I, Laville MP and Calmels T (2002) Cell type-specific localization of human cardiac S1P receptors. *J Histochem Cytochem* **50**(5):661-670.

Moreland RB, Terranova MA, Chang R, Uchic ME, Matulenko MA, Surber BW, Stewart AO and Brioni JD (2004) [3H] A-369508 ([2-[4-(2-cyanophenyl)-1-piperazinyl]-N-(3-methylphenyl) acetamide): an

MOL#61481

agonist radioligand selective for the dopamine D4 receptor. *Eur J Pharmacol* **497**(2):147-154.

Nofer JR, van der Giet M, Tolle M, Wolinska I, von Wnuck Lipinski K, Baba HA, Tietge UJ, Godecke A, Ishii I, Kleuser B, Schafers M, Fobker M, Zidek W, Assmann G, Chun J and Levkau B (2004) HDL induces NO-dependent vasorelaxation via the lysophospholipid receptor S1P3. *J Clin Invest* **113**(4):569-581.

Ohmori T, Yatomi Y, Osada M, Kazama F, Takafuta T, Ikeda H and Ozaki Y (2003) Sphingosine 1-phosphate induces contraction of coronary artery smooth muscle cells via S1P2. *Cardiovasc Res* **58**(1):170-177.

Ohta H, Sato K, Murata N, Damirin A, Malchinkhuu E, Kon J, Kimura T, Tobo M, Yamazaki Y, Watanabe T, Yagi M, Sato M, Suzuki R, Murooka H, Sakai T, Nishitoba T, Im DS, Nochi H, Tamoto K, Tomura H and Okajima F (2003) Ki16425, a subtype-selective antagonist for EDG-family lysophosphatidic acid receptors. *Mol Pharmacol* **64**(4):994-1005.

Pomposiello S, Yang XP, Liu YH, Surakanti M, Rhaleb NE, Sevilla M and Carretero OA (1997) Autacoids mediate coronary vasoconstriction induced by nitric oxide synthesis inhibition. *J Cardiovasc Pharmacol* **30**(5):599-606.

Rosen H, Gonzalez-Cabrera PJ, Sanna MG and Brown S (2009) Sphingosine 1-phosphate receptor signaling. *Annu Rev Biochem* **78**:743-768.

Ryu Y, Takuwa N, Sugimoto N, Sakurada S, Usui S, Okamoto H, Matsui O and Takuwa Y (2002) Sphingosine-1-phosphate, a platelet-derived lysophospholipid mediator, negatively regulates cellular Rac activity and cell migration in vascular smooth muscle cells. *Circ Res* **90**(3):325-332.

MOL#61481

- Salomone S, Potts EM, Tyndall S, Ip PC, Chun J, Brinkmann V and Waeber C (2008)
Analysis of sphingosine 1-phosphate receptors involved in constriction of
isolated cerebral arteries with receptor null mice and pharmacological tools. *Br J
Pharmacol* **153**(1):140-147.
- Salomone S, Yoshimura S, Reuter U, Foley M, Thomas SS, Moskowitz MA and Waeber
C (2003) S1P3 receptors mediate the potent constriction of cerebral arteries by
sphingosine-1-phosphate. *Eur J Pharmacol* **469**(1-3):125-134.
- Sanna MG, Liao J, Jo E, Alfonso C, Ahn MY, Peterson MS, Webb B, Lefebvre S, Chun
J, Gray N and Rosen H (2004) Sphingosine 1-phosphate (S1P) receptor subtypes
S1P1 and S1P3, respectively, regulate lymphocyte recirculation and heart rate. *J
Biol Chem* **279**(14):13839-13848.
- Sensken SC, Staubert C, Keul P, Levkau B, Schoneberg T and Graler MH (2008)
Selective activation of G alpha i mediated signalling of S1P(3) by
FTY720-phosphate. *Cell Signal* **20**(6):1125-1133.
- Siess W (2002) Athero- and thrombogenic actions of lysophosphatidic acid and
sphingosine-1-phosphate. *Biochim Biophys Acta* **1582**(1-3):204-215.
- Sugimoto N, Takuwa N, Okamoto H, Sakurada S and Takuwa Y (2003) Inhibitory and
stimulatory regulation of Rac and cell motility by the G12/13-Rho and Gi
pathways integrated downstream of a single G protein-coupled
sphingosine-1-phosphate receptor isoform. *Mol Cell Biol* **23**(5):1534-1545.
- Sugiyama A, Yatomi Y, Ozaki Y and Hashimoto K (2000) Sphingosine 1-phosphate
induces sinus tachycardia and coronary vasoconstriction in the canine heart.
Cardiovasc Res **46**(1):119-125.
- Takuwa Y, Okamoto Y, Yoshioka K and Takuwa N (2008) Sphingosine-1-phosphate

MOL#61481

signaling and biological activities in the cardiovascular system. *Biochim Biophys Acta* **1781**(9):483-488.

Tosaka M, Okajima F, Hashiba Y, Saito N, Nagano T, Watanabe T, Kimura T and Sasaki T (2001) Sphingosine 1-phosphate contracts canine basilar arteries in vitro and in vivo: possible role in pathogenesis of cerebral vasospasm. *Stroke* **32**(12):2913-2919.

Watterson KR, Ratz PH and Spiegel S (2005) The role of sphingosine-1-phosphate in smooth muscle contraction. *Cell Signal* **17**(3):289-298.

Yatomi Y (2006) Sphingosine 1-phosphate in vascular biology: possible therapeutic strategies to control vascular diseases. *Curr Pharm Des* **12**(5):575-587.

Zemann B, Kinzel B, Muller M, Reuschel R, Mechtcheriakova D, Urtz N, Bornancin F, Baumruker T and Billich A (2006) Sphingosine kinase type 2 is essential for lymphopenia induced by the immunomodulatory drug FTY720. *Blood* **107**(4):1454-1458.

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Footnotes

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Legends for figures

Fig. 1

Effects of S1P receptor antagonist on the S1P-induced increase in $[Ca^{2+}]_i$. A, Chemical structure of TY-52156. B, S1P₁-, S1P₂-, S1P₃-CHO or S1P₄-Chem were pretreated with vehicle or the indicated concentration (μ M) of test drug for 20 min, and then treated with vehicle (-) or S1P (0.01 μ M) to determine the increase in $[Ca^{2+}]_i$. The results are representative of three or four independent experiments. The relative percentage compared with the vehicle was calculated and expressed as mean \pm S.E.M. C, S1P₃-CHO were pretreated with vehicle or the indicated concentration (μ M) of TY-52156 for 20 min, and then treated with vehicle or the indicated concentration of S1P (logM) to determine the increase in $[Ca^{2+}]_i$. The relative percentage compared with the S1P (1 μ M, vehicle treatment) was calculated and expressed as mean \pm S.E.M. D, HUVECs were pretreated with vehicle or the indicated concentration (μ M) of the test drug for 20 min, and then treated with vehicle or SEW2871 (SEW) (5.0 μ M). The results are representative of three or four independent experiments. The ratio of the fluorescence intensity was calculated. The results are expressed as mean \pm S.E.M. * $P<0.05$, ** $P<0.01$ vs S1P alone (Dunnett's test).

Fig. 2

Selectivity of TY-52156 toward S1P₃ receptor. A, The antagonistic effect of TY-52156 was determined by a radioligand binding study based on [³H]-S1P. Results are representative of three independent experiments. The relative percentage of specific binding compared with the vehicle was calculated and expressed as mean \pm S.E.M. B, The antagonistic effect of TY-52156 was determined by a fluorescence-based Eu-GTP

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binding activity. The reaction was started by adding membranes containing human S1P₁, S1P₂, S1P₃ or S1P₅ receptors to the assay buffer including S1P (0.1 μ M) and the desired concentration of TY-52156 (TY)(μ M). The results are representative of three or four independent experiments. The relative percentage compared with the vehicle was calculated and expressed as mean \pm S.E.M. C, S1P₁-, S1P₂- or S1P₃-CHO were pretreated with vehicle, TY-52156 (TY) (10 μ M), VPC23019 (VPC) (10 μ M) or JTE013 (JTE) (1.0 μ M) for 10 min, and then treated with vehicle or S1P (0.1 μ M) to determine p44/p42 MAPK phosphorylation. D, The data obtained from three independent experiments in C were quantitatively analyzed. ## $P < 0.01$ vs S1P alone in S1P₁-CHO, ++ $P < 0.01$ vs S1P alone in S1P₂-CHO, * $P < 0.05$ vs S1P alone in S1P₃-CHO (Dunnett's test).

Fig. 3

Effects on the S1P- or U46619-induced change in cardiac coronary flow in isolated perfused rat hearts. A and B, Isolated rat hearts were perfused at 37°C in a Langendorff manner with Krebs-Henseleit bicarbonate buffer at a constant perfusion pressure (70 \pm 5 mmHg). A, S1P dose-dependently (0.001 or 0.1 μ M) decreased CF. Results are representative of five independent experiments. ** $P < 0.01$ vs vehicle (Dunnett's test). B, Perfused rat hearts were pretreated with TY-52156 (TY) (0.1 μ M), VPC23019 (VPC) (0.1 μ M) or JTE013 (JTE) (0.1 μ M) for 10 min, and then treated with vehicle, S1P (0.1 μ M) or U46619 (0.1 μ M). Results are representative of five independent experiments. ** $P < 0.01$ vs S1P alone (Dunnett's test).

Fig. 4

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Effects of TY-52156 on the S1P-induced contractile response in isolated canine cerebral arteries. A, S1P dose-dependently (0.1 to 10 μ M) induced vasoconstriction in isolated canine cerebral arteries. Results are representative of three independent experiments. B, Canine cerebral arteries were contracted by S1P (5.0 μ M). After the maximum contractile response was observed, an increasing amount of TY-52156 (up to 10 μ M) was applied to the organ chambers. Relaxation responses were measured every 10 min after addition of the indicated concentration of vehicle or TY-52156 (μ M). Results are representative of four independent experiments. * $P < 0.05$, vs indicated dose of vehicle (Student's t-test).

Fig. 5

Effects of S1P receptor antagonist on S1P-induced Rho activation in HCASMCs. HCASMCs were pretreated with vehicle or the indicated concentration (μ M) of test the drug for 10 min, and further treated with vehicle, S1P (1.0 μ M) or SEW2871 (SEW) (10 μ M) for 3 min. The data obtained from three (A, B, D) or four (C) independent experiments were quantitatively analyzed (bottom panels). * $P < 0.05$, vs S1P alone (Dunnett's test).

Fig. 6

Effects of S1P receptor antagonist on the S1P-induced increase in $[Ca^{2+}]_i$ in HCASMCs. HCASMCs were pretreated with vehicle or the indicated concentration (μ M) of the test drug for 20 min, and further treated with vehicle or S1P (0.01 μ M). Results are representative of three or four independent experiments. The relative percentage compared with the vehicle was calculated and expressed as mean \pm S.E.M. * $P < 0.05$,

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vs S1P alone (Dunnett's test).

Fig. 7

Effects of TY-52156 on FTY-720-induced bradycardia and hypertension in SD rat.

A. The oral bioavailability of TY-52156 was studied in SD rats. The plasma concentration of TY-52156 was determined by LC/MS/MS. Area under the blood concentration curve (AUC) from zero to 360 min was calculated using a trapezoidal rule. The results are expressed as mean±S.D. B, The plasma concentration of TY-52156 after oral administration was determined by LC/MS/MS. The dose of TY-52156 (TY) was 10 (*open triangle*) or 30 mg/kg (*closed triangle*). The results are expressed as mean±S.D. for three SD rats. C and D, Changes in HR (C) and SBP (D) were recorded after the oral administration of TY-52156 (TY) (10 mg/kg; *open triangle*; or 30 mg/kg; *closed triangle*) or control (*open circle*) in conscious rats. Results are representative of five independent experiments. E and F, Changes in HR (E) and MBP (F) were recorded after the administration of FTY-720 (1.0 mg/kg i.v.; *closed circle*) or control (*open circle*) in anesthetized rats. TY-52156 (TY) (10 mg/kg; *open triangle*; or 30 mg/kg; *closed triangle*) was administered orally at 4 h prior to FTY-720 injection. Results are representative of six independent experiments. * $P < 0.05$ vs control (unpaired Student's t-test). # $P < 0.05$ vs FTY-720 alone (Dunnett's test).

Fig. 8

Effects of TY-52156 on the FTY-720-P-induced increase in $[Ca^{2+}]_i$ in S1P₃-CHO. S1P₃-CHO were pretreated with vehicle or the indicated concentration (μM) of TY-52156 (TY) for 20 min, and further treated with vehicle or FTY-720-P (0.5 μM).

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The results are representative of five independent experiments. The ratio of the fluorescence intensity was calculated. The relative percentage compared with the vehicle was calculated and expressed as mean \pm S.E.M. * $P<0.05$, vs FTY-720-P alone (Dunnett's test).

Fig. 9

Summary of signaling pathways for S1P-induced vasoconstriction. S1P causes vasoconstriction in smooth muscle cells via S1P₂ and S1P₃ receptors. Inhibition of both the increase in [Ca²⁺]_i and Rho activation contributes to the efficacy of TY-52156 against S1P-induced vasoconstriction.

Fig.1

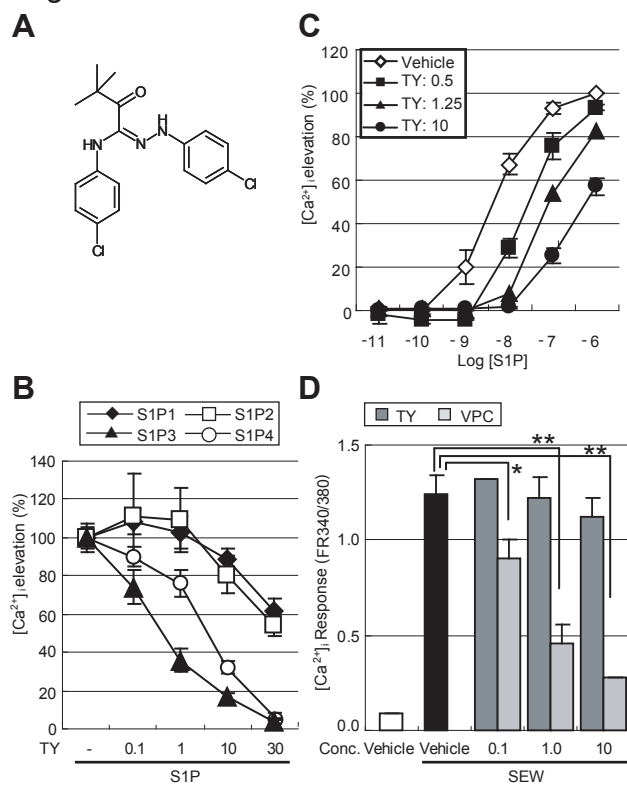


Fig.2

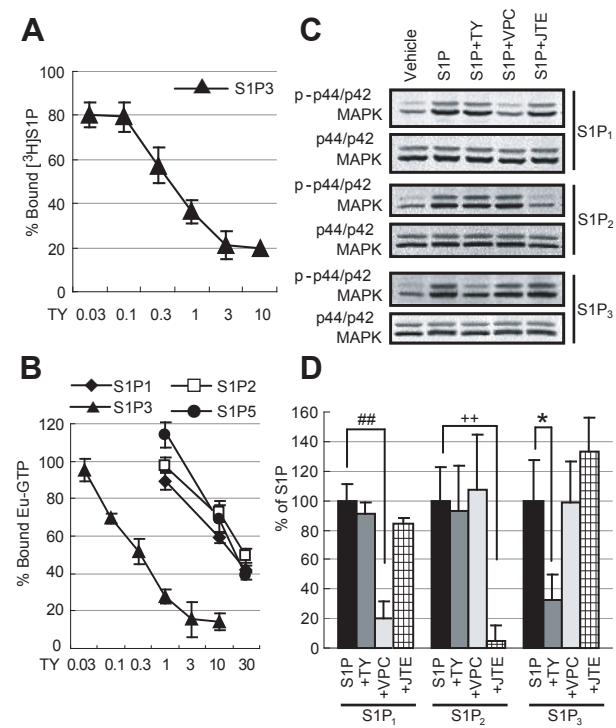


Fig.3

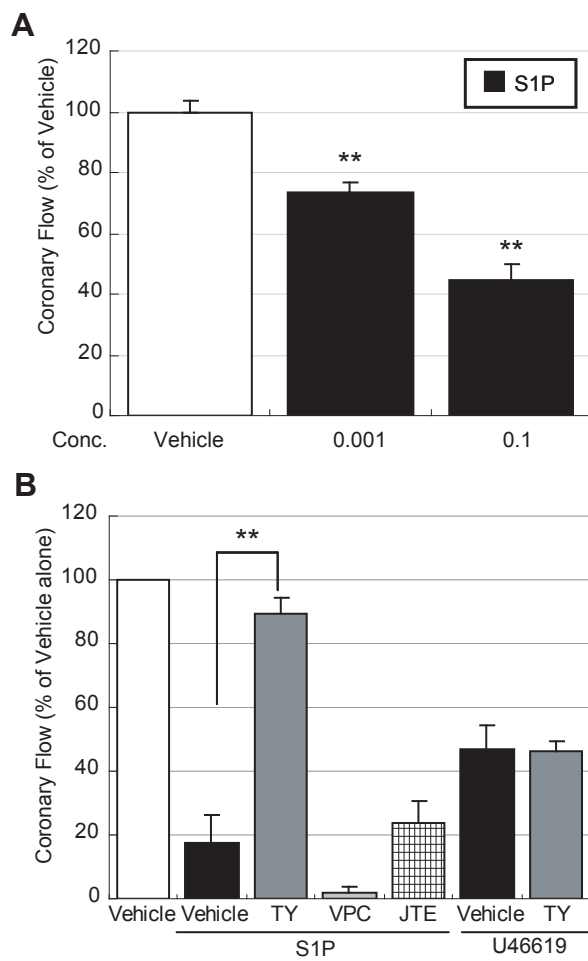


Fig.4

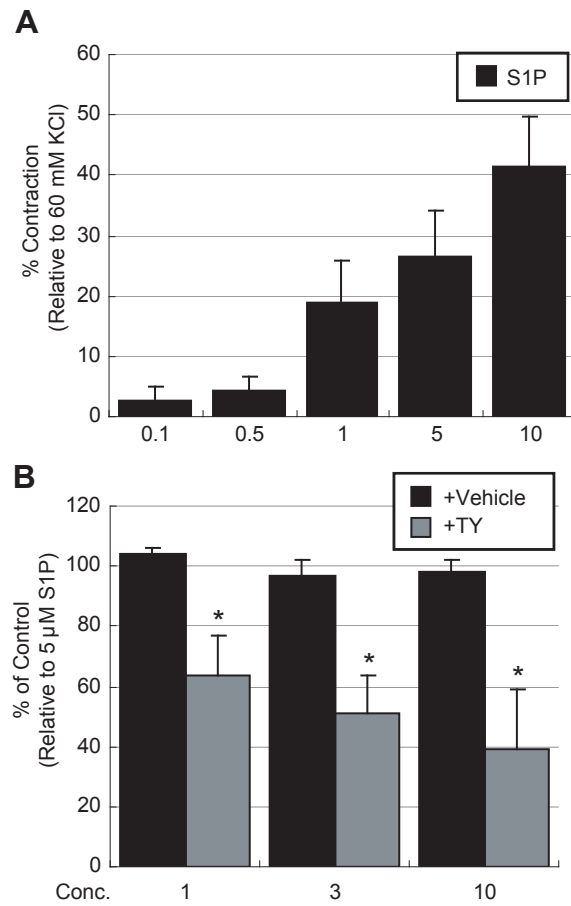


Fig.5

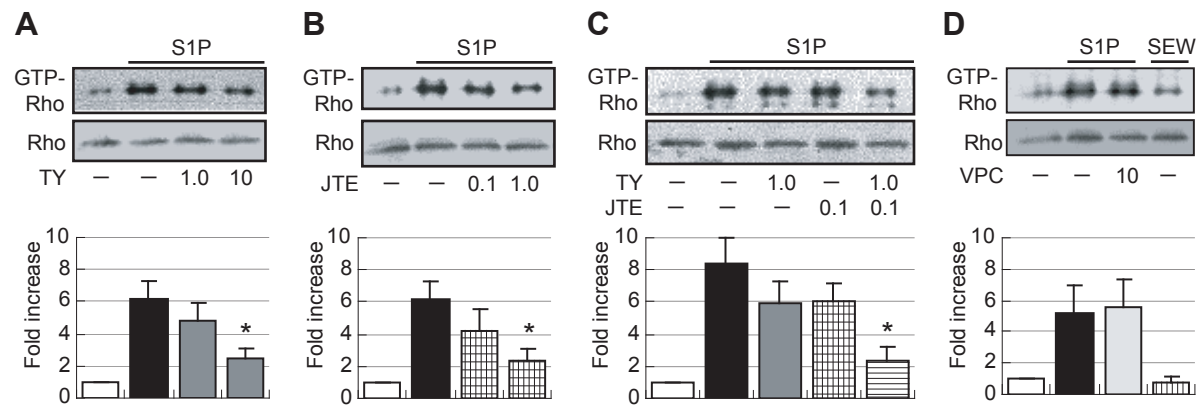


Fig.6

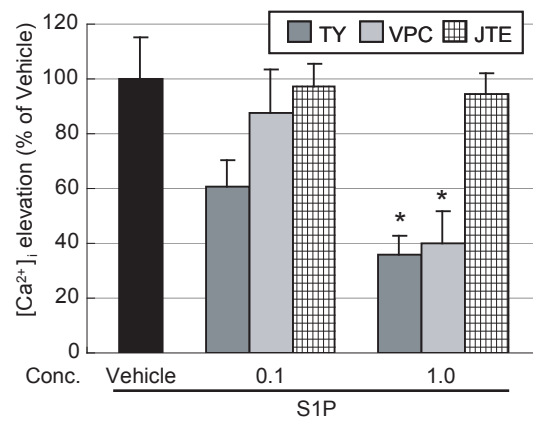


Fig.7

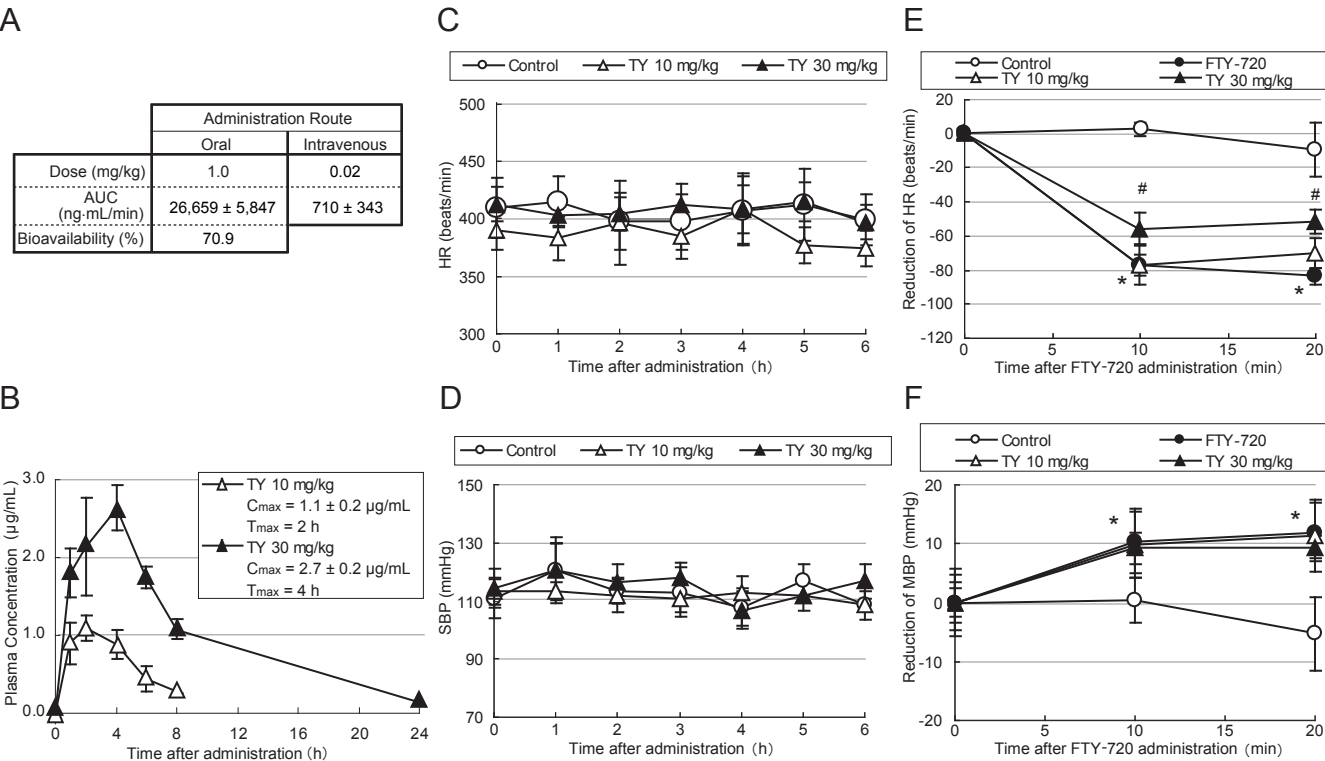


Fig.8

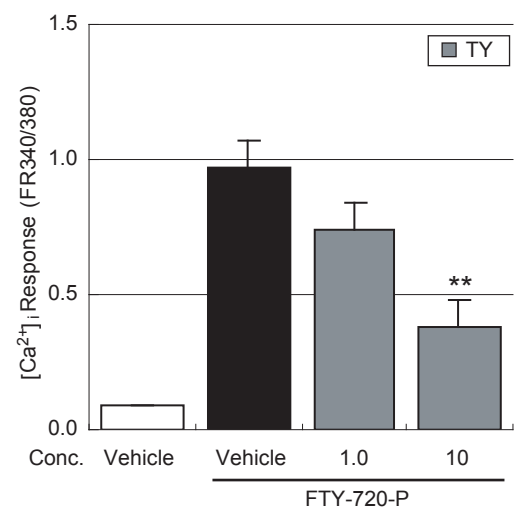


Fig.9

