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Lymphopenia induced by a novel selective $S1P_1$ antagonist structurally unrelated to S1P

Yasuyuki Fujii [a,*], Hidenori Ohtake [a], Naoya Ono [a], Toshifumi Hara [a], Takanobu Sakurai [a], Syuya Takahashi [a], Tetsuo Takayama [a], Yoshiki Fukasawa [a], Fumiyasu Shiozawa [a], Narutoshi Tsukahara [c], Takehiro Hirayama [c], Yasuyuki Igarashi [b], Ryo Goitsuka [c]

[a] Department of Molecular Function and Pharmacology Laboratories, Taisho Pharmaceutical Co. Ltd., 1-403 Yoshino-cho, Saitama-shi, Saitama 331-9520, Japan
[b] Laboratory of Biomembrane and Biofunctional Chemistry, Hokkaido University, Sapporo, Hokkaido 060-0812, Japan
[c] Division of Development and Aging, Research Institute for Biological Sciences, Tokyo University of Science, 2669 Yamazaki, Noda, Chiba 278-0022, Japan

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ABSTRACT

Sphingosine 1-phosphate (S1P) regulates lymphocyte trafficking via type-1 S1P receptor ($S1P_1$) and participates in many pathological conditions. We developed a novel type $S1P_1$-selective antagonist, TASP0251078, which is structurally unrelated to S1P. This competitive antagonist inhibited binding of S1P to $S1P_1$ resulting in reduced signaling downstream of $S1P_1$, including GTPγS-binding and cAMP formation. TASP0251078 also inhibited S1P-induced cellular responses such as chemotaxis and receptor-internalization. Furthermore, when administered *in vivo*, TASP0251078 induced lymphopenia in blood, which is different from previously reported effects of other $S1P_1$-antagonists. In a mouse contact hypersensitivity model, TASP0251078 effectively suppressed ear swelling, leukocyte infiltration, and hyperplasia. These findings provide the chemical evidence that $S1P_1$ antagonism is responsible for lymphocyte sequestration from the blood, and suggest that the effect of $S1P_1$ agonists on lymphocyte sequestration results from their functional antagonism.

1. Introduction

Lymphocyte egress from primary and secondary lymphoid tissues is dependent on the lipid mediator sphingosine 1-phosphate (S1P), which induces this effect by binding to $S1P_1$, one of the five members of S1P receptors [1–5]. FTY720, an immunosuppressive agent, induces lymphopenia by sequestering lymphocytes inside lymphoid organs through its agonistic activity on $S1P_1$ [6–10]. However, the mechanisms underlying this effect remain controversial, and two different models have been proposed for this effect. One model, referred to as functional antagonism, is that some agonists, such as phosphorylated FTY720, bind directly to $S1P_1$ on lymphocytes themselves and cause receptor internalization, creating an $S1P_1$-null state on lymphocytes, which in turn prevents S1P dependent lymphocyte chemotaxis along a gradient of endogenous S1P and prevents migration from the lymph node[11–13]. In support of this hypothesis, lymphocytes pretreated with FTY720 show reduced $S1P_1$ expression and chemotaxis *in vitro* [11,12] and lymphocytes from $S1P_1$-deficient mice fail to egress from thymus, spleen, and lymph nodes [11,14]. The other model is that ligand binding to $S1P_1$ on endothelial cells results in endothelial cytoskeletal alterations that blocks lymphocyte egress. This model is based on two-photon microscopic observations of lymph node explants, which show that lymphocyte movement into lymphatic sinuses is unaffected by an $S1P_1$ antagonist, but is inhibited by an $S1P_1$ agonist and this inhibition is reversed by addition of the antagonist [15,16].

Here, we developed a novel $S1P_1$ antagonist structurally different from S1P and showed that this antagonist induces lymphopenia by competitive inhibition of S1P binding to $S1P_1$. Furthermore, it acts as an immunosuppressant similar to the $S1P_1$ agonist FTY720. Our data thus support the model that $S1P_1$ agonists behave as functional antagonists to induce lymphocyte sequestration from the circulation.

2. Materials and methods

2.1. Chemicals

(D)-Boc-alanine methyl ester was used as a starting material for TASP0251078 synthesis. (D)-Boc-alanine methyl ester was treated

Abbreviations: S1P, sphingosine 1-phosphate; $S1P_1$, type 1 sphingosine 1-phosphate receptor; CHS, contact hypersensitivity response; HEK293, human embryonic kidney cell line; CHO-K1, Chinese hamster ovary cell line; HTS, high-throughput screening; GTP, guanosine-5′-triphosphate; GDP, guanosine-5′-diphosphate; HEPES, N-2-Hydroxyethylpiperazine-N′-2-Ethanesulfonic Acid; BSA, bovine serum albumin; cAMP, cyclic adenosine monophosphate; TNCB, 2,4,6-trinitrochlorobenzene; IC_{50}, half maximal (50%) inhibitory concentration; TC_{50}, half maximal (50%) toxic concentration; EIA, Enzyme-Linked ImmunoSorbent Assay

* Corresponding author at: 1-403 Yoshino-cho, Saitama-shi, Saitama 331-9530, Japan. Fax: +81 48 669 7254.
E-mail address: y.fujii@po.rd.taisho.co.jp (Y. Fujii).

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with hydrazine to give *tert*-butyl (1-hydrazinyl-1-oxopropan-2-yl) carbamate. The hydrazide was reacted with ethyl isothioisocyanate, and the resulting *tert*-butyl {1-[2-(ethylcarbamothioyl)hydrazinyl]-1-oxopropan-2-yl}carbamate was then subjected to base-catalyzed cyclization to obtain *tert*-butyl [1-(4-ethyl-5-sulfanyl-4*H*-1,2,4-triazol-3-yl)ethyl]carbamate, which was readily S-methylated using iodomethane to give *tert*-butyl {1-[4-ethyl-5-(methylsulfanyl)-4*H*-1,2,4-triazol-3-yl]ethyl}carbamate. The methyl thioether derivative was oxidized using m-chloroperbenzoic acid and the resulting *tert*-butyl {1-[4-ethyl-5-(methylsulfonyl)-4*H*-1,2,4-triazol-3-yl]ethyl}-carbamate was subjected to deprotection of the amino group using trifluoroacetic acid to give 1-[4-ethyl-5-(methylsulfonyl)-4*H*-1,2,4-triazol-3-yl]ethanamine trifluoroaceticacid salt. The trifluoroacetic acid salt was treated with sodium methoxide to provide 1-(4-ethyl-5-methoxy-4*H*-1,2,4-triazol-3-yl)ethanamine, which was reacted with 3-chloro-4-fluoro-benzenesulfonylchloride to obtain TASP0251078 (3-chloro-*N*-[(1*R*)-1-(4-ethyl-5-methoxy-4*H*-1,2,4-triazol-3-yl)ethyl]-4-fluorobenzenesulfonamide) as colorless powder (Chemical Purity: 98.8% Optical purity: >99%e.e.) S1P was purchased from Biomol (NY, USA).

2.2. Cell lines

Cell line HEK293 cells stably expressing Flag epitope-tagged human (h) $S1P_1$, $hS1P_3$, $hS1P_4$, or $hS1P_5$ were generated by transfecting HEK293 cells with the pCMV-tag2 plasmid containing full-length cDNAs for $hS1P_1$, $hS1P_3$, $hS1P_4$, or $hS1P_5$ using a Lipofectamine 2000 reagent (Invitrogen. CA, USA), followed by selection of drug-resistant clones in the presence of 0.5 mg/ml G418. Expression of hS1P receptors on these clones was confirmed by flow cytometry analysis with anti-Flag M2 monoclonal antibody using a flow cytometer EPICS® XL-MCL (Coulter Co. FL, USA). CHO-K1 cells stably expressing mouse (m) S1P receptors, including $mS1P_1$, $mS1P_2$, $mS1P_3$, and $mS1P_4$, were generated, as previously described [17].

2.3. Competitive ligand binding assay

Membranes prepared from HEK293 cells and CHO cells expressing S1P receptors were incubated with ^{33}P-labeled S1P (3000 Ci/mmol) in the presence or absence of various concentration of TASP0251078 for 60 min at room temperature, and then the reaction was terminated by collecting the membranes onto GF/C filter plates using a Packard Filtermate Universal Harvester. The filter-bound radioactivity was counted using a TopCount NXT Microplate Scintillation and Luminescence Counter C384V01J (PerkinElmer. MA, USA). Specific binding was calculated by subtracting radioactivity that remained in the presence of 1000-fold excess of unlabeled S1P. The binding constant (K_i) associated with the ligand-receptor interaction was determined from the IC_{50} using the Chang–Prusoff equation ($K_i = IC_{50}/(1 + [L]/K_d)$. In applying this equation, the concentration of ^{33}P-labeled ligand (L) is 0.1 nM and the K_d value was 0.15 nM in our assay.

2.4. [^{35}S]-GTPγS binding assay

The HEK293 cells stably expressing $hS1P_1$ were homogenized with assay buffer (50 mM HEPES buffer, pH 7.5, containing 100 mM NaCl, 5 mM $MgCl_2$, 20 μM GDP), and centrifuged at 45,000×*g* for 20 min at 4 °C. Aliquots of pellets containing membrane fractions (5 μg) were pre-incubated with GTP-binding buffer (50 mM HEPES buffer, pH 7.5, containing 100 mM NaCl, 5 mM $MgCl_2$, 20 μM GDP, 0.1% BSA, 20 μg/ml saponin) in the presence or absence of various concentrations of S1P or TASP0251078 for 30 min at 30 °C. [^{35}S]-GTPγS (1200 Ci/mmol; 0.1 nM) was added and incubated for 30 min at 30 °C. The reaction was then terminated by rapid filtration under vacuum through a UniFilter GF/C microplate. The filter-bound activity was counted using a TopCount NXT Microplate Scintillation and Luminescence Counter C384V01J. Nonspecific binding was determined in the presence of 30 μM GTPγS. Schild regression plot was analyzed using linear regression, and pA_2 (negative logarithm of the concentration of antagonist causing a dose ratio = 2) values determined.

2.5. Measurement of cAMP formation

The HEK293 cells expressing $S1P_1$ were seeded in 96-well plates at a density of 1×10^5 cells per well and grown for 1 day. After replacing to the fresh serum-free medium, the cells were pre-incubated with medium containing 0.5 mM 3-isobutyl-1-methylxanthine for 20 min at 37 °C, and further incubated for 10 min in the medium containing 10 nM S1P with or without various concentrations of TASP0251078. The cells were then stimulated with 10 μM forskolin for 15 min at 37 °C, and cAMP levels were determined using a cAMP enzyme immunoassay (EIA) system (Amersham-Pharmacia Biotech, Inc. NJ, USA), according to the manufacturer's protocol.

2.6. Immunocytochemistry

The HEK293 cells expressing $S1P_1$ were cultured overnight on Poly-D-lysine-coated 8-well chamber slides (BD Bioscience. NJ, USA) in DMEM with 10% FCS, After 6 h, serum starvation in DMEM containing 0.1% fatty acid-free BSA, cells were treated in the same medium with vehicle or 100 nM S1P in the presence or absence of 10 μM TASP0251078 for 30 min. The resultant cells were fixed with 4% paraformaldehyde, and stained with rabbit polyclonal anti-Flag antibody (Cell Signaling Technology. MA, USA), followed by goat anti-rabbit IgG Alexa Fluor555 (Invitogen. CA, USA). Images were acquired with a laser scanning confocal microscope (Leica SPII).

2.7. Chemotaxis assay

Migration assays were performed in 96-well transwell chambers with 8-μm polycarbonate membrane filters (Neuroprobe. MD, USA). Cells were serum starved in F-12 nutrient mixture containing 0.1% fatty acid-free BSA (Sigma. MO, USA) for 30 min and plated in triplicate into the top chamber at the density of 5×10^4 per well, and the bottom chamber contained various concentrations of TASP0251078 with 10 nM S1P. Cells were allowed to migrate for 4 h in a humidified chamber at 37 °C with 5% CO_2. Serum-free medium was used as a control. After the incubation, cells in the upper and bottom wells were harvested and counted using the ATPlite-M Luminescence Assay System (PerkinElmer. MA, USA).

2.8. In vivo pharmacokinetic and biological analyses

Female Balb/c mice (5–7 weeks old) and male Lewis rats (6–8 weeks old), purchased from Charles River (Kanagawa, Japan), were orally administered the indicated dosages of TASP0251078 or vehicle (5% Arabic gum). Blood samples of rats were collected from tail vein after administration of TASP0251078. Blood samples of mice were collected from caudal vena cava for pharmacokinetic analyses and external carotid artery for biological analyses after administration of TASP0251078. Plasma was separated by centrifugation of blood samples at 11,200×*g* for 3 min at 4 °C and stored at −80 °C until assayed. Full blood cell counts were determined with the ADVIA120 hematology system calibrated for mouse blood (Siemens Healthcare Diagnostics Inc. MA, USA). The plasma concentrations of TASP0251078 were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS) with negative ion electrospray ionization (ESI) following protein precipitation. Briefly, an aliquot (50 μL) of plasma samples was mixed with 200 μL of organic solvent (acetonitrile /methanol (9:1, v/v)) containing an internal standard (I.S.). After centrifugation at 4300×*g* for 15 min, the resulting