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Sphingosine-1-phosphate (S1P) displays sustained $S1P_1$ receptor agonism and signaling through S1P lyase-dependent receptor recycling

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ABSTRACT

The sphingosine-1-phosphate (S1P) type 1 receptor ($S1P_1R$) is a novel therapeutic target in lymphocyte-mediated autoimmune diseases. $S1P_1$ receptor desensitization caused by synthetic $S1P_1$ receptor agonists prevents T-lymphocyte egress from secondary lymphoid organs into the circulation. The selective $S1P_1$ receptor agonist ponesimod, which is in development for the treatment of autoimmune diseases, efficiently reduces peripheral lymphocyte counts and displays efficacy in animal models of autoimmune disease. Using ponesimod and the natural ligand S1P, we investigated the molecular mechanisms leading to different signaling, desensitization and trafficking behavior of $S1P_1$ receptors. In recombinant $S1P_1$ receptor-expressing cells, ponesimod and S1P triggered Gαi protein-mediated signaling and β-arrestin recruitment with comparable potency and efficiency, but only ponesimod efficiently induced intracellular receptor accumulation. In human umbilical vein endothelial cells (HUVEC), ponesimod and S1P triggered translocation of the endogenous $S1P_1$ receptor to the Golgi compartment. However, only ponesimod treatment caused efficient surface receptor depletion, receptor accumulation in the Golgi and degradation. Impedance measurements in HUVEC showed that ponesimod induced only short-lived Gαi protein-mediated signaling followed by resistance to further stimulation, whereas S1P induced sustained Gαi protein-mediated signaling without desensitization. Inhibition of S1P lyase activity in HUVEC rendered S1P an efficient $S1P_1$ receptor internalizing compound and abrogated S1P-mediated sustained signaling. This suggests that S1P lyase – by facilitating S1P1 receptor recycling – is essential for S1P-mediated sustained signaling, and that synthetic agonists are functional antagonists because they are not S1P lyase substrates.

1. Introduction

Sphingosine-1-phosphate (S1P) is a bioactive lipid with a variety of important physiological roles in the cardiovascular and immune system [1–3]. S1P effects are mediated through activation of a family of five G protein-coupled receptors ($S1P_1$–$S1P_5$), of which the $S1P_1$ receptor is the most extensively investigated S1P receptor subtype to date. $S1P_1$ receptors are expressed in many cell types including lymphocytes and endothelial cells. $S1P_1$ receptors that are activated by S1P induce Gαi protein-mediated signaling and regulate many different cellular responses, such as cell migration or modulation of cell–cell contacts. In endothelial cells, $S1P_1$ stimulation by S1P leads to increased tight junction formation thereby reducing vascular permeability [4–6]. In lymphocytes, $S1P_1$ receptor activity promotes their exit from secondary lymphoid organs along a gradient of increasing S1P concentration into the efferent lymphatics, a crucial step in lymphocyte trafficking between tissue and blood. The importance of $S1P_1$ receptor functionality in lymphocyte circulation has been supported by the findings that $S1P_1$ receptor-deficient lymphocytes, lymphocytes of animals that were treated with synthetic $S1P_1$ receptor antagonists, and lymphocytes from animals or humans that were treated with synthetic $S1P_1$ receptor agonists do not egress from secondary lymphoid organs [7–10]. The latter finding has been explained by "functional antagonism" that is exerted by synthetic $S1P_1$ receptor agonists due to $S1P_1$ receptor desensitization [11,12]. The lymphocyte count reducing activities of synthetic $S1P_1$ receptor agonists have been studied extensively using compounds such as the non-selective $S1P_{1,2,3,4,5}$ agonist FTY720-P or its non-phosphorylated pro-drug FTY720 (Gilenya™, fingolimod) [13–16]. In recombinant and non-recombinant cell types, $S1P_1$ receptors were readily internalized after activation by FTY720-P and remained internalized, thereby creating $S1P_1$ receptor depleted cells that were refractory to further $S1P_1$ receptor stimulation [17–22]. It was furthermore shown

Abbreviations: BSA, bovine serum albumin; CHO, Chinese hamster ovary; CRC, concentration–response curve; EC_{50}, half maximimal excitatory concentration; E_{max}, maximal efficacy versus a defined control; FBS, fetal bovine serum; GPCR, G protein coupled receptor; GRK, GPCR related kinase; HUVEC, human umbilical vein endothelial cells; PBS, phosphate buffered saline; S1P, sphingosine-1-phosphate; S1PR, sphingosine-1-phosphate receptor; SPL, S1P lyase; SD, arithmetic standard deviation; σ_g, geometric standard deviation.

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that synthetic agonists imposed a different fate on $S1P_1$ receptors compared to the natural ligand S1P. For example, FTY720-P, but not S1P, induced $S1P_1$ receptor poly-ubiquitination, prolonged internalization and proteasomal degradation [18,22–24]. In addition, $S1P_1$ receptors that were activated by FTY720-P were claimed to remain internalized and to continue to signal via Gαi protein-mediated pathways, even after ligand wash-out. This phenomenon was termed "persistent signaling" and was not seen with the natural ligand S1P [25].

The ability of synthetic $S1P_1$ receptor agonists, but not S1P, to efficiently induce $S1P_1$ receptor internalization and desensitization, is believed to be a key feature of "functional antagonism" and lymphocyte trapping, but the reasons for this differential receptor modulation are still unknown. It has been suggested that ligands impose different receptor conformations resulting in different downstream receptor modifications, such as phosphorylation and poly-ubiquitination [12,18,25,26]. However, alternative explanations, such as differential stability of natural and synthetic ligands to intracellular degradation, could also be possible [27–30].

Following the approval of the first non-selective $S1P_{1,2,3,4,5}$ receptor agonist FTY720 (Gilenya™, fingolimod) for the treatment of multiple sclerosis, several novel $S1P_1$ receptor agonists with improved selectivity profiles entered clinical development for the treatment of autoimmune diseases [31]. Ponesimod, a representative of this new class of selective $S1P_1$ receptor agonists, was shown to reduce circulating lymphocytes in vivo and displayed disease modifying activities in animal models of autoimmune diseases [32,33].

Using recombinant and native $S1P_1$ receptor expressing cell systems, we show here that ponesimod is a potent and efficient inducer of transient $S1P_1$ receptor signaling, leading to receptor internalization, intracellular receptor accumulation, desensitization and ultimately degradation, whereas the natural ligand S1P causes sustained signaling, induces limited intracellular receptor accumulation, and does not desensitize the $S1P_1$ receptor system. Ponesimod and S1P both induced $S1P_1$ receptor trafficking to the Golgi compartment in human umbilical vein endothelial cells (HUVEC). However, the efficiency of $S1P_1$ receptor trapping in the Golgi was much lower for S1P- compared to ponesimod-stimulated receptors. We show here that the almost complete lack of $S1P_1$ receptor retention upon S1P stimulation was caused by ligand susceptibility to the degrading enzyme S1P lyase, a feature that is not shared with synthetic $S1P_1$ agonists such as ponesimod. These results suggest that S1P lyase (SPL) is not only important for control of S1P levels in lymph and blood [34], but also plays an important role in the physiological $S1P_1$ receptor recycling process.

2. Materials and methods

2.1. Pharmacological compounds

S1P and W146 were purchased from Avanti Polar Lipids, Inc. (Alabaster, Alabama, USA). Ponesimod and the S1P lyase blocker (compound 2b [35]) were synthesized by Actelion Pharmaceuticals (Allschwil, Switzerland). FTY720-P was purchased from Cayman Chemical (Estonia). GT-11 was purchased from Matreya (Pleasant Gap, PA, USA).

2.2. Cell culture

HeLa cells stably expressing human $S1P_1$ receptors were cultured in RPMI 1640 containing fetal calf serum (FCS) and 1 mg/ml geneticin. CHO-$S1P_1$ PathHunter® cells for β-arrestin assays (DiscoveRx, UK) were cultured in Ham's F12 containing 10% FBS, 100 U Penicillin, 1 μg/ml streptomycin, 300 μg/ml hygromycin and 800 μg/ml geneticin. HUVEC (Cascade Biologics, UK) were cultivated on gelatin-coated plates in Medium 200 (Invitrogen, Switzerland) supplemented with Low Serum Growth Factor and used between passage 8 and 12.

2.3. Cyclic AMP assay

Cells were seeded into 96-well plates, grown overnight, washed with assay buffer (1× HBSS, 20 mM Hepes, 0.0375% $NaHCO_3$, 0.1% fatty acid free BSA) and then subjected to agonist dilution series in assay buffer in the presence of IBMX (0.5 mM) and forskolin (10 μM). After 30 min, cells were lysed, cAMP levels were determined using the Tropix cAMP-screen System (Applied Biosystems, USA) and luminescence was read with a microplate reader (Synergy4, BioTek Instruments, USA). EC_{50} values were calculated using the proprietory IC50 witch software (Actelion Pharmaceuticals Ltd., Switzerland).

2.4. $S1P_1$ β-arrestin recruitment assay

CHO-$S1P_1$ PathHunter® cells were detached and seeded in 384-well plates and grown overnight in OptiMEM medium (Invitrogen, Switzerland). Compounds were incubated for 10 min, 60 min or 240 min at 37 °C. After addition of the Flash detection reagent, the luminescence was read using a microplate reader (Synergy4, BioTek Instruments, USA). EC_{50} values were calculated using the proprietary IC50 witch software (Actelion Pharmaceuticals Ltd., Switzerland).

2.5. Analysis of $S1P_1$ receptor internalization by flow cytometry

Recombinant HeLa-$S1P_1$ cells were seeded into 12-well plates and the agonist dilution series was added in low serum medium (0.5% FBS). After incubation with agonists for indicated times, the cells were washed with PBS and detached with cell dissociation buffer, then $S1P_1$ receptors were stained with mouse anti-h$S1P_1$R antibodies (R&D systems, USA), diluted in PBS/2 mM EDTA and 0.5% fatty acid free BSA (Calbiochem, Germany). As secondary antibody we used Alexa Fluor 488 goat anti-mouse IgG (Life Technologies, Switzerland). Then, propidium iodide-negative cells were analyzed using the flow cytometer FACSAria IIu (BD Biosciences, USA).

2.6. Immunofluorescence microscopy

HeLa-$S1P_1$ cells and HUVEC were seeded into gelatin-coated 8-chamber slides (BD Falcon, USA) and grown overnight. $S1P_1$ receptor agonists were added and incubated for the indicated times. Cells were fixed (3% paraformaldehyde), permeabilized and stained in PBS, 10% FBS, 0.1% saponin (Sigma Aldrich, Switzerland) using rabbit anti-h$S1P_1$ receptor antibodies (Santa Cruz Biotechnology, USA), mouse anti-human lamp1 antibodies (BD Biosciences, USA) and anti-GM130 antibodies (BD Biosciences, USA). Secondary reagents were Alexa Fluor 488 goat anti-rabbit (Life Technologies, Switzerland) and Alexa Fluor 546 goat-anti mouse (Life Technologies, Switzerland). Nuclei were stained with Hoechst 33342 (Life Technologies, Switzerland). Samples were analyzed using the Zeiss Axiovert 200 immunofluorescence microscope (Zeiss, Germany). SPL co-localization analysis was performed in HUVEC using the following primary antibodies: rabbit anti-hSGPL1 (Atlas antibodies, Sweden), mouse anti-human lamp1 (BD Biosciences, USA), mouse anti-human EEA1 (BD Biosciences, USA), mouse anti-human ERp72 (BD Biosciences, USA) and anti-GM130 (BD Biosciences, USA). Secondary reagents were Alexa Fluor 568 goat anti-rabbit (Life Technologies, Switzerland) and Alexa Fluor 488 goat-anti mouse (Life Technologies). Samples were analyzed by confocal microscopy (Leica TCS SPE DMI 4000B; Leica, Germany). The localization of internalized fluorescent S1P was performed by incubating NBD-S1P (omega(7-nitro-2-1,3-benzoxadiazol-4-yl)-D-erythro-S1P; Avanti Polar Lipids, Inc., USA) for 1 h at 37 °C with HUVEC followed by extensive washing. Fixed and permeabilized cells were then stained with anti-GM130 antibodies and goat-anti-mouse Alexa Fluo 568 (Life Technologies) secondary reagent and Hoechst 33342. Images were taken using the Zeiss Axiovert 200 microscope.