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FTY720 (S)-Phosphonate Preserves S1PR1 Expression and Exhibits Superior Barrier Protection to FTY720 in Acute Lung Injury

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Abstract

Objective—Effective therapies are needed to reverse the increased vascular permeability that characterizes acute inflammatory diseases such as acute lung injury (ALI). FTY720 is a pharmaceutical analog of the potent barrier-enhancing phospholipid, sphingosine 1-phosphate (S1P). Because both FTY720 and S1P have properties that may limit their usefulness in patients with ALI, alternative compounds are needed for therapeutic use. The objective of this study is to characterize the effects of FTY720 (S)-phosphonate (Tys), a novel analog of FTY720-phosphate, on parameters of pulmonary vascular permeability in vitro and alveolar-capillary permeability in vivo.

Setting—University-affiliated research institute.

Subjects—Cultured human pulmonary endothelial cells (EC); C57BL/6 mice.

Interventions—EC were stimulated with S1P receptor 1 (S1PR1) agonists to determine effects on S1PR1 expression. ALI was induced in C57BL/6 mice with bleomycin to assess effects of S1PR1 agonists.

Measurements and Main Results—Tys potently increases human pulmonary EC barrier function in vitro as measured by transendothelial electrical resistance (TER). Reduction of S1P receptor 1 (S1PR1) with siRNA significantly attenuates this TER elevation. Tys maintains endothelial S1PR1 protein expression in contrast to >50% reduction after incubation with S1P, FTY720, or other S1PR1 agonists. Tys does not induce β -arrestin recruitment, S1PR1 ubiquitination, and proteosomal degradation that occur after other agonists. Intraperitoneal administration of Tys every other day for 1 week in normal or bleomycin-injured mice maintains

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significantly higher lung S1PR1 expression compared with FTY720. FTY720 fails to protect against bleomycin-induced ALI in mice, while Tys significantly decreases lung leak and inflammation.

Conclusion—FTY720 (*S*)-phosphonate is a promising barrier-promoting agent that effectively maintains S1PR1 levels and improves outcomes in the bleomycin model of ALI.

Keywords

vascular permeability; endothelium; ubiquitination; ARDS; GPCR; bleomycin

Introduction

Acute inflammatory diseases such as acute lung injury/acute respiratory distress syndrome (ALI/ARDS) disrupt endothelial barrier integrity, cause a sustained increase in vascular permeability, and have high mortality (1, 2). Despite recent studies demonstrating survival benefits of optimal low tidal volume ventilator management in ARDS, mortality rates remain excessive (>30%) with an estimated 75,000 deaths per year in the US (2). Effective therapies for preserving or reconstituting the vascular barrier are lacking, but their successful development may help reverse this pathophysiologic process.

The protective role of platelets in maintenance of vascular barrier integrity has long been known (3, 4), with the endogenous signaling phospholipid, sphingosine 1-phosphate (S1P), identified as the critical barrier-protective product responsible for this effect (5, 6). Intravenous administration of S1P significantly reduces LPS-induced pulmonary microvascular leakage in both murine and canine models of ALI (7, 8). S1P signaling via the S1P receptor 1 (S1PR1) pathway is critical for barrier function maintenance and improvement (1). Through S1PR1 ligation, S1P not only increases baseline barrier integrity but also effectively protects the endothelium from the barrier disruptive effects of edemagenic agents such as thrombin (5). FTY720 (2-amino-2-[2-(4-octylphenyl)ethyl]propane-1,3-diol) (Gilenya®), a structural analogue of the S1P precursor sphingosine (9), is an FDA-approved therapy for multiple sclerosis (10) that has similar barrier protective effects as S1P *in vitro* (11, 12) and in the mouse model of LPS-induced ALI (8).

Despite their impressive potential, S1P and FTY720 produce a series of side effects that may limit their usefulness in ALI patients, such as bradycardia and increased airway hyperresponsiveness (10, 13–15). In addition, FTY720 functions as an immunosuppressant, which makes it useful in multiple sclerosis (10, 16) but possibly detrimental in patients with ALI, many of whom have sepsis or infection as a triggering event (16). Both S1P and FTY720 also exhibit barrier disruption at higher concentrations (17). Based on these limitations, we sought to develop new FTY720 analogs, such as (*S*)-FTY720-phosphonate (Tys), in hope of identifying better therapeutic agents (17). Prior work has demonstrated that Tys induces rapid TER elevation (5–10 min) with more potent effects on maximal barrier enhancement compared to S1P and FTY720 *in vitro* (17). Importantly, Tys remains barrier protective even at the higher concentrations at which S1P and FTY720 cause barrier disruption (17). Moreover, in LPS-induced lung injury in mice, Tys demonstrates significant

protective effects without altering peripheral blood leukocyte and lymphocyte levels compared with treatment with LPS alone, suggesting that Tys may not induce immunosuppression like FTY720 (17).

In the current report, we further characterize the effect of Tys compared to other S1PR1 agonists on S1PR1 expression *in vitro* and *in vivo* and on bleomycin-induced ALI in mice, a model selected for the ability to produce an inflammatory lung injury persisting for several weeks. This allows for evaluation of prolonged responses that better mimic the time course of human ALI (18). In addition, a recent study demonstrated that prolonged exposure to FTY720 increased lung vascular leak and mortality in bleomycin-injured mice (19), in contrast with prior reports indicating protective effects of FTY720 in ALI murine models of shorter duration (e.g., LPS) (8, 17, 20). Our results demonstrate that Tys uniquely maintains S1PR1 expression level relative to other agonists without activating the β -arrestin/ubiquitin pathway, while providing superior protection against bleomycin-induced ALI in mice compared to FTY720. These data suggest that further investigation of Tys is warranted as a potential therapeutic agent in lung injury syndromes.

Materials and Methods

Reagents

Unless otherwise specified, reagents were obtained from Sigma (St. Louis, MO). (*S*)-FTY720-phosphonate ((3*S*)-3-(amino)-3-(hydroxymethyl)-5-(4'-octylphenyl)-pentylphosphonic acid, Tys) and (*R*)-FTY720-phosphonate (1R) were synthesized as previously described (21). FTY720 (Fingolimod, Gilenya) and (*S*)-FTY720-phosphate (p-FTY720) were kindly provided by Novartis. Other sources: S1P (Biomol, Plymouth Meeting, PA), SEW2871 (Cayman Chemical, Ann Arbor, MI), SB649146 (Glaxo Smith Kline, King of Prussia, PA), rabbit anti-S1PR1 and mouse anti-ubiquitin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), bleomycin (APR Pharmaceuticals, Schaumburg, IL).

Cells

Human pulmonary artery endothelial cells (HPAEC) and lung microvascular endothelial cells (HLMVEC) were purchased from Lonza (Walkersville, MD) and cultured as previously described (12). TangoTM EDG1-*bla* U2OS cells were cultured as per the supplier's instructions (Invitrogen).

Transendothelial monolayer electrical resistance (TER)

Cells were seeded in polycarbonate wells containing evaporated gold microelectrodes in EBM-2 with 2% FBS for 24 h until confluent. TER measurements were performed using an electrical cell-substrate impedance sensing system (ECIS) (Applied Biophysics, Troy, NY) as previously described (5, 22). TER values from each microelectrode were pooled at discrete time points and plotted vs. time as the mean $\pm$ S.E.M.

Small interference RNA (siRNA)

Negative control siRNA #2 (D-001810-02) and S1PR1 siRNA (L-003655-00-0005) were purchased from Dharmacon. HPAEC (70% confluent) were transfected with 100 nM siRNA

using DharmaFECT 1 per manufacturer's protocol. Silencing efficacy was assessed by Western blot after 72 h.

Western blotting and Immunofluorescence

These assays were performed per standard procedures exactly as previously described (12).

Immunoprecipitation

700 µg of cell lysates (in 500 µl of RIPA buffer) were immunoprecipitated with 3 µg of anti-S1PR1 antibody by rotation overnight. The beads were washed with lysis buffer 3 times, boiled in SDS-sample buffer at 95 °C for 5 min, and proteins were separated in 4–15% SDS PAGE gels.

β-arrestin activation

The Invitrogen Tango™ EDG1--*bla* U2OS Cell-based Assay was used to determine β-arrestin activation. Briefly, EDG1-*bla* U2OS cells were grown in FreeStyle™ Expression Medium for 48 h. Then, 1 µM of S1P, Tys, 1R, FTY720, p-FTY720, or 10 µM of SEW was added, and the cells were incubated in a humidified 37°C/5% CO₂ incubator for 5 h. Fluorescence substrates were added, and the cells were kept at room temperature in the dark for 2 h. Fluorescence intensity was detected and the blue/green emission ratio for each well was calculated and used as the S1PR1 activation indicator. Experiments were repeated 3 times, and the final ratio was expressed as the mean ± S.E.M.

Bleomycin ALI models

All experiments and animal care procedures were approved by the University of Illinois at Chicago Animal Care and Use Committee. Male C57BL/6 (20–25 g) mice 8–10 weeks old were purchased from Jackson Laboratory (Bar Harbor, ME). C57BL/6 mice received a single intratracheal dose of bleomycin at 0.6 U/kg (or sterile saline) on Day 0 followed immediately by intraperitoneal injection of Tys (0.5 mg/kg), FTY720 (0.5 mg/kg), or saline. Additional doses of Tys or FTY720 were injected on Days 3 and 6. Bronchoalveolar lavage (BAL) fluid and lungs were then collected on Day 7. BAL fluid was used to detect BAL protein levels, WBC count, and WBC differential count. Lungs were perfused with saline to remove blood for Western blot, tissue albumin, and histopathology evaluation. Peripheral blood was obtained on Day 7 for examination of total cell counts and lymphocytes. Experiments were repeated 3 times. 6–10 mice were used per experimental group.

BAL protein accumulation, leukocyte quantification and tissue albumin

BAL was performed by flushing the lungs with 1 ml of cold HBSS through the tracheal cannula, as previously described (23). The recovered lavage fluid (~0.8 ml) was centrifuged (500×g for 20 min), and the cell pellet was resuspended in 200 µl of ice-cold HBSS. Cells were counted using a TC 10 automated cell counter machine (Bio-Rad). BAL differential cell count was prepared using cytocentrifugation (Cytospin 3; Shandon Instruments, Pittsburgh, PA), stained with Diff-Quik (Dade Behring, Düringen, Switzerland), and determined using morphologic criteria under a light microscope, with approximately 400

cells counted per slide. Tissue albumin concentration was determined by using Mouse Albumin ELISA Kit (E99–134, Bethyl Laboratories, Montgomery, TX).

Lung histology

Lung inflammatory morphology alteration was characterized as previously described (23). Excised left lungs (3–4 animals/group) were placed immediately in formalin overnight, followed by embedding in paraffin for histological evaluation by hematoxylin–eosin staining. Levels of leukocyte infiltration were calculated to indicate inflammatory injury severity in all study groups as previously described (23).

Statistics

Student's *t*-test was used to compare the means of data from two different experimental groups. One-way Anova and nonparametric Tukey's test were performed for multiple group comparisons. Results are expressed as means $\pm$ S.E.M., with significance level predetermined as $P < 0.05$.

Results

S1PR1 mediates EC barrier enhancement by Tys *in vitro*

S1PR1 is the primary receptor responsible for mediating S1P-induced EC barrier enhancement *in vitro* and reduced vascular leak *in vivo* (5, 23). Prior work demonstrated that the (*S*)-phosphonate analog of FTY720 (Tys) exhibits a wider barrier protective range and greater potency than either S1P or FTY720 (17). To determine the role of S1PR1 in Tys-induced EC barrier enhancement, HPAEC permeability was assessed by measuring transendothelial resistance (TER), a highly sensitive *in vitro* measurement of barrier function (5). Reduction of S1PR1 expression via specific siRNA (Inset, Fig. 1A) decreased basal TER (data not shown) and significantly inhibited barrier enhancement by Tys (Fig. 1A). Similarly, SB649146, a specific inverse agonist of S1PR1 (24), significantly inhibited barrier enhancement by Tys (Fig. 1B). These data strongly indicate that S1PR1 is essential for maximal TER elevation by Tys, consistent with the prior observation that Tys activates S1PR1 but not the other four S1P receptors (25).

Tys maintains S1PR1 protein expression compared to other agonists

Because S1PR1 function is critical for the maintenance of barrier integrity, we next determined the effect of Tys on S1PR1 expression relative to other S1PR1 agonists in cultured HPAEC. HPAEC were stimulated for 4 h with one of the following S1PR1 agonists: S1P, FTY720, p-FTY720, SEW-2871 (SEW), Tys, or the (*R*)-enantiomer of Tys (1R). Optimal barrier enhancing concentrations for each compound were used based upon preliminary TER measurements (data not shown). S1PR1 protein expression (determined by Western blotting) was down-regulated significantly in HPAEC stimulated with S1P, FTY720, 1R, p-FTY720, or SEW (Fig. 2). In contrast, Tys maintained S1PR1 expression at basal levels (Fig. 2A–B). Thus, Tys is unique among these S1PR1 agonists in that it preserves receptor expression while still enhancing endothelial barrier function as well or better than the other compounds (17).

S1PR1 agonists induce proteasomal degradation of the receptor

Proteasomes are the major sites for intracellular protein degradation (26) and participate in S1PR1 degradation by p-FTY720 (27). We next determined if a similar proteasomal mechanism mediates decreased S1PR1 expression induced by other ligands. HPAEC were pretreated with the proteasome specific inhibitor, MG132 (10 μ M), for 2 h and then were stimulated with barrier-enhancing concentrations of Tys, 1R, or p-FTY720 for 4 h. MG132 treatment did not alter S1PR1 expression basally or after Tys stimulation, but it significantly inhibited 1R- or p-FTY720-induced S1PR1 degradation (Fig. 2C–D). These results suggest that the proteasome is the major pathway for S1PR1 degradation after agonist stimulation.

Tys fails to induce ubiquitination of S1PR1

Ubiquitination of proteins is a common pre-requirement before proteins enter proteasomes and are degraded (28). This post-translational modification occurs on S1PR1 during p-FTY720-induced degradation of the receptor (27, 29). We next examined S1PR1 ubiquitination levels in HPAEC after challenge with S1P, FTY720, p-FTY720, SEW, Tys, or 1R. S1PR1 was immunoprecipitated from HPAEC lysates by S1PR1 antibody, and then ubiquitination of the receptor was detected by Western blotting. S1P and p-FTY720 significantly increased ubiquitination of S1PR1 within 1 h (Fig. 3A–B), whereas FTY720, SEW, and 1R had little effect at this time point (data not shown). At 2 h of incubation all of these compounds significantly increased ubiquitination of the S1PR1 receptor (Fig. 3C–D). In contrast, Tys failed to induce S1PR1 ubiquitination at either time point.

Reduced β -arrestin recruitment to S1PR1 after Tys

β -arrestin plays a critical role in trafficking of nearly all G protein-coupled receptors (30) and is involved in p-FTY720-induced S1PR1 degradation (27). The recruitment of β -arrestin to S1PR1 by S1P, FTY720, Tys, 1R, p-FTY720, and SEW was examined using the Tango S1PR1-bla U2OS Cell-based Assay in which β -arrestin binding to S1PR1 is detected by fluorescence signaling. S1P, 1R, FTY720, p-FTY720, and SEW all significantly induced recruitment of β -arrestin to S1PR1 (by $\sim$ 8–20 fold) (Fig. 4). In contrast, Tys stimulated only a small increase in β -arrestin recruitment, even at concentrations as high as 50 μ M (Fig. 4).

Immunofluorescent studies were performed to assess the subcellular localization of S1PR1 in human lung EC. In unstimulated confluent HLMVEC and HPAEC, S1PR1 was observed at the periphery along cell-cell junctions (Fig. 5A, Suppl. Fig. 1A). S1P or FTY720 stimulation resulted in a redistribution of S1PR1 to discrete intracellular areas with a loss of peripheral staining (Fig. 5C–D, Suppl. Fig. 1C–D). However, Tys-challenged EC exhibited a peripheral pattern of S1PR1 immunofluorescence similar to control EC (Fig. 5B, Suppl. Fig. 1B). These results are consistent with the β -arrestin data and indicate that Tys, in contrast to other agonists, does not induce significant internalization or downregulation of S1PR1.

Tys preserves S1PR1 expression *in vivo* and reduces bleomycin-induced murine ALI

To explore the effects of Tys on S1PR1 expression *in vivo*, we next determined S1PR1 protein levels in mice lungs. C57BL/6 mice received intraperitoneal injections of vehicle, FTY720, or Tys on days 0, 3, and 6. Lungs were harvested on day 7, and S1PR1 expression

was detected in the lung homogenates by Western blotting. Tys maintained S1PR1 expression near control levels, while FTY720 significantly reduced S1PR1 expression by ~50% (Fig. 6A–B).

Next, we determined if Tys preserves S1PR1 expression in bleomycin-injured mice. Mice received a single dose of bleomycin 0.6 u/kg (IT) to induce ALI and then were given either vehicle, FTY720, or Tys on days 0, 3, and 6. Mouse lungs were harvested on day 7. Consistent with both *in vitro* data (Fig. 2) and uninjured animal data (Fig. 6A–B), FTY720 significantly reduced S1PR1 expression compared to control and bleomycin-alone animals (Fig. 6C–D). In contrast, S1PR1 lung expression in Tys-treated mice was similar to control and bleomycin-alone animals (Fig. 6C–D). Moreover, evaluation of circulating WBC levels in these animals demonstrated a similar trend. In control or bleomycin-injured mice, blood counts collected on day 7 revealed no significant differences between mice receiving vehicle or Tys, while both total WBC and lymphocyte counts were decreased in FTY720-treated mice (Table 1). Since the immunosuppressive effects of FTY720 are due to its ability to deplete peripheral lymphocytes by downregulating their S1PR1 receptors (31–33), these data further support the hypothesis that Tys maintains cell S1PR1 expression at higher levels than FTY720.

To determine if Tys-mediated maintenance of S1PR1 lung expression correlated with improved ALI outcomes, we examined the effects of FTY720 and Tys on multiple indices of pulmonary alveolar-capillary leak and inflammatory injury in bleomycin-injured mice. In uninjured mice, there were no differences in BAL protein level, total cell count, or PMNs in vehicle control, FTY720-, and Tys-treated mice after 7 days (Fig. 7A–C). Although bleomycin increased BAL protein level, total cell count, PMNs, and lung tissue albumin in all groups after 7 days, Tys was significantly protective relative to the other conditions (Fig. 7A–D). Moreover, FTY720-treated mice exhibited increased BAL protein levels compared to control bleomycin-injured mice, consistent with prior reports that FTY720 worsens some parameters in this model (19). Histological analysis of lung sections from these bleomycin-injured mice demonstrated decreased influx of PMNs into the tissue of Tys-treated mice compared to vehicle controls and FTY720-treated animals (Fig. 8). Thus, these *in vivo* results confirm that Tys, unlike FTY720, maintains S1PR1 expression in lung tissue during prolonged exposure and produces superior protection against bleomycin-induced ALI.

Discussion

Multiple studies over the past decade have demonstrated that S1P/S1PR1 signaling is critical in maintaining and regulating endothelial barrier function (1, 5, 23, 34–37). Cumulatively, these prior studies strongly suggest that agonists capable of inducing sustained activation of S1PR1 may have therapeutic benefit in vascular leak syndromes. Because S1P and FTY720 produce effects likely to limit their utility in ALI syndromes (e.g., airway hyperresponsiveness, bradycardia, immunosuppression, and barrier disruption at high concentrations) (10, 13–15), new analogs are being developed in the search for better therapeutic agents. FTY720 (S)-phosphonate (Tys) is a novel analog of FTY720 and S1P that exhibits superior EC barrier promoting properties *in vitro* (17). The results of our current study indicate that silencing of S1PR1 or inhibition of S1PR1 by its inverse agonist

significantly inhibits TER elevation by Tys, indicating that S1PR1 is critical in Tys-induced barrier enhancement (Fig. 1).

Interestingly, all of the S1PR1 ligands investigated in this study (S1P, 1R, FTY720, p-FTY720, and SEW), except for Tys, induce ubiquitination (Fig. 3) and decreased protein expression of S1PR1 (Fig. 2). Even the enantiomer of Tys, 1R, produces these effects. The mechanistic importance of ubiquitination pathways in the pathogenesis of ALI is being increasingly recognized (38, 39). Prior work has demonstrated that p-FTY720 ligates S1PR1 and, although initially barrier promoting, induces β -arrestin mediated ubiquitination, internalization and degradation of S1PR1 that eventually leads to increased vascular leak (27, 29). Our data now strongly suggest that this pathway is invoked by the majority of S1PR1 ligands as S1P, p-FTY720, FTY720, 1R, and SEW all induce significant β -arrestin recruitment to S1PR1 (Fig. 4), S1PR1 internalization (Fig. 5), ubiquitination of S1PR1 (Fig. 3), and degradation of S1PR1 in proteasomes (Fig. 2). In sharp contrast to the other agonists studied here, Tys maintained S1PR1 expression without significantly increasing β -arrestin binding with S1PR1, S1PR1 internalization, or ubiquitination of S1PR1 (Fig. 2, 3, 4, 5).

Since S1PR1 is critical for ligand-induced barrier improvement as well as maintenance of basal barrier function (1, 5, 23), the ability of Tys to maintain S1PR1 expression suggests that Tys may be a more effective agent for promoting persistent S1PR1 activation (40–42) and reversing alveolar-capillary leak than other S1PR1 agonists. Animal studies confirm the importance of S1PR1 expression and signaling in the treatment of ALI in vivo. For example, in a mouse model of influenza infection, the S1PR1 specific agonist CYM-5442 suppressed virally induced cytokine storm and mortality through endothelial-mediated S1PR1 signaling (43). The importance of S1PR1 expression in modulating lung injury is further supported by the observation that S1P failed to preserve or enhance lung barrier function in LPS-stimulated S1PR1+/- mice (23). In another study, FTY720 at a single dose of 0.5 mg/kg induced partial degradation of S1PR1 and a moderate increase in vascular permeability in mice after 24 h, while a higher concentration of FTY720 (5 mg/kg) induced dramatic S1PR1 degradation and a massive increase in vascular permeability (29). In addition, FTY720 concentrations in this range exacerbated ventilator-induced ALI in mice (44) as well as pulmonary leak and mortality in bleomycin-injured mice (19).

In the current study, the bleomycin model of ALI was employed because prolonged duration of injury was necessary to investigate our hypothesis that S1P agonists that maintain S1PR1 expression (e.g., Tys) will exhibit superior protection to those that decrease S1PR1 expression over time (e.g., FTY720). Our results indicate that FTY720 significantly decreased S1PR1 lung expression (Fig. 6), while Tys maintained S1PR1 at control levels. Most importantly, Tys significantly inhibited bleomycin-induced alveolar-capillary leakage and inflammatory cell recruitment (Fig. 7–8), while FTY720 failed to protect against these ALI-associated indices. These data are consistent with our recent report that Tys is protective against radiation-induced lung injury, while FTY720 is not (45). Thus, multiple recent reports including ours have described increased ALI in mice receiving FTY720, indicating that this compound is unlikely to be beneficial as a therapeutic agent for vascular leak syndromes. In contrast, we report here that Tys shows superior protection than FTY720 in bleomycin-induced ALI.

It is important to consider some limitations of our current findings. Although we have emphasized the differential effects of S1PR1 agonists on vascular endothelial receptor expression and permeability, the intratracheal model of bleomycin-induced ALI targets the alveolar epithelium as the site of initial injury (18). Because alveolar epithelial cells express S1PR1, it is theoretically possible that Tys is exerting some of its superior protective effects on alveolar permeability in addition to the vascular endothelium. Our animal model data do not allow for precise discrimination between the effects of Tys on these two cell barriers. Another limitation of our study is that detailed pharmacokinetic information for Tys is not yet available, so the every third day dosing regimen used in our *in vivo* protocol may not be optimal. Finally, although multiple lines of evidence support the hypothesis that Tys is mediating its barrier enhancing effects through S1PR1 signaling, the possibility remains that some portion of Tys effects may be mediated via another mechanism.

The mechanism that mediates specific S1PR1 signaling by Tys is still unknown and remains under investigation. Classical agonists of G protein-coupled receptors bind and stabilize receptor conformation in a manner that activates multiple downstream pathways (46). However, an increasing number of studies have demonstrated that there are biased ligands that stabilize only conformations that activate specific signaling effects, e.g., G protein-biased or β -arrestin-biased (40–42). Such bias agonism clearly broadens the definition of ligand action and provides the opportunity for more selective targeting of beneficial signaling while reducing or avoiding unwanted downstream effects (41). One possibility is that Tys may induce differential conformational changes in the receptor compared with the other ligands tested here. This specific conformation may allow Tys to activate Gi signaling and downstream cytoskeletal changes to produce barrier enhancement (17) while avoiding the β -arrestin-mediated signaling down-regulation pathway that leads to ubiquitination and degradation (Suppl. Fig. 2). In a recent mutagenesis study of S1PR1, structural amino acid motifs necessary for its activation by p-FTY720 were characterized, but these elements did not participate in targeting the receptor for degradation (47). The structural elements responsible for determining whether activated S1PR1 is recycled to the membrane or targeted for degradation remain unclear. Recent work describing the crystal structure of S1PR1 (48, 49) may help facilitate additional structure/function studies of the differential conformational changes induced in the receptor by various ligands.

Conclusions

Tys augments EC barrier function *in vitro* through S1PR1 ligation. However, Tys does not induce the recruitment of β -arrestin to S1PR1, ubiquitination of S1PR1, or subsequent degradation of S1PR1, which allows Tys to maintain normal expression of S1PR1. Tys preserves S1PR1 expression both *in vitro* and *in vivo* while FTY720 induces significant degradation of the receptor. Most importantly, Tys significantly protects against bleomycin-induced lung injury while FTY720 fails to do so. As a result of this S1PR1-protective characteristic of Tys, it may prove to be a superior agent for reversing vascular leak syndromes, and further studies are warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. S1PR1 mediates HPAEC barrier enhancement by Tys *in vitro*

A, HPAEC transfected with S1PR1 siRNA (si-S1PR1) or control siRNA (sic) were plated on gold microelectrodes and then stimulated with 1 μ M Tys. Western blot (inset) demonstrates S1PR1 silence effect. **B**, HPAEC were pretreated with 10 μ M SB649146 for 1 h and then stimulated with 1 μ M Tys as indicated by arrows. The TER tracings represent pooled data ($\pm$ S.E.M) from 3 independent experiments.

Figure 2. Tys maintains S1PR1 protein expression compared to other agonists

Fig. A, B: HPAEC were stimulated with vehicle control (c), S1P, FTY720, Tys, 1R, p-FTY720 (each at 1 μ M), or SEW (10 μ M) for 4 h. S1PR1 expression level was detected by Western blot. **A**, representative western blot; **B**, Bar graph represents pooled densitometry from 3 independent experiments. **Fig. C, D:** HPAEC were pretreated with 20 μ M MG132 (MG) (a proteasome inhibitor) for 2 h, and then stimulated with vehicle control (C), Tys, 1R, or p-FTY720 (1 μ M) for 4h. S1PR1 expression level was detected by Western blot. **C**, representative western blot; **D**, Bar graph represents pooled densitometry from 3 independent experiments. *, $p < 0.01$ vs. control (C); #, $p < 0.01$ compared to 1R without MG; &, $p < 0.05$ compared to p-FTY720 without MG.

Figure 3. Tys does not induce ubiquitination of S1PR1

HPAEC were stimulated with vehicle control (c), S1P, Tys, or p-FTY720 (1 μ M) for 1 h (**A** and **B**) or with FTY720, Tys, 1R, (1 μ M) or SEW (10 μ M) for 2 h (**C** and **D**). S1PR1 was immunoprecipitated by S1PR1 antibody, and ubiquitination of S1PR1 was detected by Western blotting with ubiquitin antibody. **A**, **C**: representative western blot; **B**, **D**: Bar graph represents pooled densitometry from 3 independent experiments. *, $p < 0.05$ compared to control or Tys.

Figure 4. β -arrestin recruitment to S1PR1 after Tys is decreased compared to other agonists
 Quiescent Tango™ EDG1-bla U2OS Cells were stimulated with S1P, 1R, FTY720, p-FTY720 (each at 1 μ M), 10 μ M SEW (10 μ M) or 0.01–50 μ M Tys as indicated for 5 h. After another 2 h-incubation with the fluorescence substrate, the blue/green fluorescence intensity was detected, and the blue/green emission ratio was used as the recruit degree indicator per the manufacturer's protocol. Results are expressed as means $\pm$ S.E.M from 3 independent experiments. *, $p < 0.01$ compared to control; #, $p < 0.01$ compared to other agonists.

Figure 5. S1P and FTY720, but not Tys, induce internalization of S1PR1

Confluent HLMVEC grown on glass bottom culture dishes were stimulated with vehicle control, Tys, FTY720, or S1P (1 μ M) for 2 h. Cells were then fixed and immunostained with S1PR1 antibody per standard protocol. Arrows indicate that S1PR1 localizes to the cell periphery in control and Tys-treated cells, while S1P and FTY720 induce internalization of the receptor. Representative figures from 3 independent experiments are shown.

Figure 6. Tys preserves S1PR1 expression in mouse lungs *in vivo*

A, Mice received saline control (c), FTY720 (0.5 mg/kg, IP) or Tys (0.5 mg/kg, IP) on days 0, 3, and 6. Lungs homogenates were collected on day 7 for detection of S1PR1 expression (and actin loading control) by Western blot. A representative Western blot is shown. **B**, Bar graph represents pooled densitometry from 3 experiments. N = 5 for control, and 10 for the other conditions. *, $p < 0.01$. **C**, Mice received bleomycin (0.6 U/kg, IT) to induce lung inflammation and then were treated with saline control, FTY720 (0.5 mg/kg, IP), or Tys (0.5 mg/kg, IP) on days 0, 3, and 6. Lungs homogenates were collected on day 7 for detection of S1PR1 expression by Western blot. A representative Western blot is shown. **D**, Bar graph represents pooled densitometry from 3 experiments. N = 5 for control, and n = 8 for the other conditions. *, $p < 0.01$.

Figure 7. Tys is protective in bleomycin-induced ALI

Mice received bleomycin (0.6 U/kg, IT) to induce lung inflammation and then were treated with saline control, FTY720 (0.5 mg/kg, IP), or Tys (0.5 mg/kg, IP) on days 0, 3, and 6. BAL samples were collected on day 7. BAL protein levels (A), BAL total leukocyte counts (B), BAL percentage PMNs (C) and lung tissue albumin (D) were performed as described in Methods. N = 6–9 per group. *, $p < 0.01$.

Figure 8. Tys attenuates lung tissue leukocyte infiltration in bleomycin-injured mice

Sections from the lungs of mice which received bleomycin and FTY720 or Tys were stained with hematoxylin-eosin for histologic evaluations of lung inflammation and leukocyte infiltration. **A**, Representative lung images from mice with indicated interventions. **B**, Relative levels of leukocyte infiltration into the lungs, quantified as aggregated histologic scores as described in Methods. N = 4–5 per group. *, $p < 0.01$.

Table 1

Total cell and lymphocyte counts in blood from bleomycin-challenged mice treated with Tys, FTY720, or Control (C).

Treatment	C	Control Tys	FTY720	Bleo	Bleomycin Bleo + Tys	Bleo + FTY720
WBCs #	7.332 ± 0.6	6.999 ± 0.4	*4.551 ± 0.3	**2.795 ± 0.2	3.024 ± 0.3	***1.567 ± 0.1
Lymphs #	6.278 ± 0.5	5.883 ± 0.3	*3.464 ± 0.3	**1.050 ± 0.1	2.136 ± 0.3	***0.874 ± 0.1

Expressed as (10³/uL) the mean +/- SEM for N=6-9, ANOVA non parametric Tukey's test.

* Denotes p<0.001 vs C and Tys groups

** Denotes p<0.001 vs C, FTY720, and Tys groups

*** Denotes p<0.05 vs Bleo+Tys