Sphingosine-1-phosphate receptor-independent lung endothelial cell barrier disruption induced by FTY720 regioisomers

Sara M. Camp1, Alexander Marciniak2, Eddie T. Chiang1, Alexander N. Garcia3, Robert Bittman4,*, Robin Polt2, Ruth G. Perez5, Steven M. Dudek6 and Joe G. N. Garcia1

1Department of Medicine, The University of Arizona, Tucson, AZ, USA; 2Department of Chemistry and Biochemistry, The University of Arizona, Tucson, AZ, USA; 3Department of Radiation Oncology, The University of Arizona, Tucson, AZ, USA; 4Department of Chemistry and Biochemistry, Queens College of the City University of New York, Flushing, NY, USA; 5Department of Molecular and Translational Medicine, Graduate School of Biomedical Sciences, Center of Emphasis in Neuroscience, Paul L. Foster School of Medicine, Texas Tech University Health Sciences Center, El Paso, TX, USA; 6Division of Pulmonary and Critical Care Medicine, Department of Medicine, University of Illinois at Chicago, Chicago, IL, USA

Abstract

Rationale: Vascular permeability is a hallmark of acute respiratory distress syndrome (ARDS) and ventilator-induced lung injury pathobiology; however, the mechanisms underlying this vascular dysregulation remain unclear, thereby impairing the development of desperately needed effective therapeutics. We have shown that sphingosine-1-phosphate (S1P) and 2-amino-2-(2-([4-octylphenyl]ethyl))-1,3-propanediol (FTY720) analogues are useful tools for exploring vascular barrier regulation mechanisms.

Objective: To experimentally define the effects of FTY720 regioisomers on lung endothelial cell barrier regulation.

Methods: Specific barrier-regulatory receptor and kinase inhibitors were utilized to probe signaling mechanisms involved in FTY720 regioisomer-mediated human lung endothelial cell barrier responses (trans-endothelial electrical resistance, TER). Docking simulations with the S1P1 receptor were performed to further evaluate FTY720 regioisomer signaling.

Results: FTY720 regioisomers produced potent endothelial cell barrier disruption reflected by declines in TER alterations. Pharmacologic inhibition of Gi-coupled S1P receptors (S1P1, S1P2, S1P3) failed to alter FTY720 regioisomer-mediated barrier disruption; findings that were corroborated by docking simulations demonstrating FTY720 regiosomers were repelled from S1P1 docking, in contrast to strong S1P1 binding elicited by S1P. Inhibition of either the barrier-disrupting PAR-1 receptor, the VEGF receptor, Rho-kinase, MAPK, NFkB, or PI3K failed to alter FTY720 regioisomer-induced endothelial cell barrier disruption. While FTY720 regioisomers significantly increased protein phosphatase 2 (PP2A) activity, PP2A inhibitors failed to alter FTY720 regioisomer-induced endothelial cell barrier disruption.

Conclusions: Together, these results imply a vexing model of pulmonary vascular barrier dysregulation in response to FTY720-related compounds and highlight the need for further insights into mechanisms of vascular integrity required to promote the development of novel therapeutic tools to prevent or reverse the pulmonary vascular leak central to ARDS outcomes.

Keywords

FTY720, sphingosine 1-phosphate, regioisomer, endothelial, permeability, acute respiratory distress syndrome

Introduction

Sustained vascular barrier leak is a critical contributor to the morbidity and mortality observed in acute inflammatory diseases, such as acute respiratory distress syndrome...
(ARDS) and sepsis. For critically ill patients experiencing respiratory failure as a result of ARDS, an inflammatory lung syndrome with high mortality rate of 30–40%,1–3 reversal of the diminished pulmonary vascular barrier integrity is an important clinical goal. Increases in lung vascular leakage, inflammatory cell influx, and inflammatory cytokine expression are all hallmarks of ARDS pathology.4,5; however, mechanisms underlying ARDS are still unclear and effective therapeutics targeting the vasculature are still needed.

Disruption of lung vascular endothelial cell (EC) monolayer integrity leads to respiratory failure due to flooding of interstitial and alveolar compartments with fluid, protein, and inflammatory cells.6 Effective therapeutic agents to prevent or reverse inflammation-mediated vascular barrier leak are lacking.7 We previously demonstrated the potent barrier-enhancing properties of the endogenous phospholipid sphingosine 1-phosphate (SIP), the related pharmaceutical agent 2-amino-2-(2-[4-octylphenyl]ethyl)-1,3-propanediol (FTY720), and several novel synthetic analogs of FTY720 including (S)-FTY720-phosphonate (or TySIPonate)8–11 in models of inflammatory lung injury. SIP, a sphingolipid produced by multiple cell types, initiates a series of downstream effects through the ligation of the G_i-coupled S1P1 receptor (SIP1), culminating in enhancement of the EC cortical actin ring, improved cell–cell and cell–matrix interactions, and increased barrier function in vitro.10,12,13 Subsequently, the SIP1 receptor has also been proven to be protective in in vivo murine models of acute lung injury.14 The pharmaceutical agent FTY720, a structural analog of SIP, potently enhances lung EC barrier function via G_i-coupled receptor signaling.9,15 Phosphonate and ene-phosphonate analogs of FTY720, such as TySIPonate, demonstrate similar but not identical barrier regulatory properties to SIP and FTY720.8 Oxazolo-oxazole derivatives of FTY720 reduce EC permeability induced by LPS or TNFα in vitro.16 Moreover, SIP, FTY720, and TySIPonate attenuate lipopolysaccharide (LPS)-induced preclinical lung injury.6,17,18 More recent studies19 examined additional FTY720 analogs, where TER and labeled dextran studies demonstrate that (R)-methoxy-, fluoro-, and β-glucuronide FTY720 analogs display in vitro barrier-enhancing properties comparable or superior to FTY720 and SIP due to SIP1-dependent receptor ligation. Thus, SIP, FTY720, and various analogs (such as TySIPonate) represent a novel class of agents that are potential therapeutic options for addressing the increased vascular permeability observed in inflammatory lung diseases such as ARDS.

However, both SIP and FTY720 exhibit specific characteristics that imply limited therapeutic utility in ARDS patients. The endogenous ligand SIP exhibits a limited therapeutic window with higher concentrations (>5 μM) increasing in vitro lung EC permeability,8 and intratracheal SIP administration, producing pulmonary edema in vivo via ligation of the abundant SIP3 receptor on epithelium. This results in disruption of the epithelial barrier20 and produces contraction of human airway smooth muscle cells,21 increased airway hyper-responsiveness in mice,22 and cardiac toxicity via SIP3 activation in the heart.23,24 While FTY720 is an FDA-approved therapy for multiple sclerosis due to its effectiveness as an immunosuppressant via down-regulated SIP1 signaling,25,26 this immunosuppressive effect may be harmful in critically ill patients with sepsis or other infectious disease processes. In addition, multiple studies have recently demonstrated detrimental effects on vascular permeability of higher concentrations and prolonged exposure to FTY720.27 Higher concentrations of FTY720 produce tissue edema28 and exacerbate both ventilator-induced lung injury29 and bleomycin-induced lung injury11,27 in preclinical models. The barrier-disruptive effects of high concentrations of FTY720 are likely mediated through down-regulation of endothelial SIP1 expression and subsequent increased permeability in the absence of SIP1 ligation and signaling.11,28 TySIPonate, unlike FTY720, does not down-regulate SIP1 expression and, therefore, remains highly protective in preclinical models of bleomycin-induced lung injury.11

Another concern when considering this class of sphingolipids as potential therapeutic agents in the critically ill is that subtle changes in the chemical structure produce dramatically paradoxical effects. In contrast to vascular protective effects of (R)-methoxy-FTY720, the (S)-methoxy-FTY720 regioisomer disrupts lung EC barrier integrity in association with actin stress fiber formation and robust intracellular Ca_2+ release but independent of myosin light chain or ERK phosphorylation.19 More importantly, the (S)- and (R)-FTY720 regioisomers or positional isomer analogs,8 analogs of FTY720 with the same molecular formula but with altered functional group positions, exhibit exactly opposing effects on EC barrier regulation. At modest concentrations, FTY720 is EC barrier-enhancing, whereas the (S)- and (R)-FTY720 regioisomers potently disrupt EC barrier integrity as monitored by TER and flux of labeled dextran.8

In the current study, we sought to enhance understanding of the mechanistic basis for (S)- and (R)-FTY720 regioisomer-mediated vascular permeability and characterize the mechanisms by which this novel class of agents modulate EC permeability.

**Methods**

*Synthesis of FTY70 regioisomers*

Regioisomers of FTY720 (S enantiomer or 3S; R enantiomer or 3R) were synthesized as previously described.30

**Reagents**

SIP was purchased from Sigma-Aldrich (St. Louis, MO), and FTY720 was generously provided by Novartis (Basel, Switzerland) or purchased from AbMole BioScience (Kowloon, Hong Kong, China) for phosphatase assays.
SIP1 inverse agonist SB649146 was generously provided by Glaxo Smith Kline (King of Prussia, PA). NFkB, okadaic acid, SIP2, and SIP3 inhibitors were purchased from Cayman Chemicals (Ann Arbor, MI). MAPK, PI3K, PKC, Rho-kinase, and VEGER2 inhibitors, in addition to pertussis toxin (PTX), were purchased from EMD Millipore Corporation (Billericia, MA). PAR-1 neutralizing antibody and the Calyculin A inhibitor were purchased from Santa Cruz Biotechnology (Dallas, TX). PP2A catalytic subunit (PP2Ac, Cayman Chemical), malachite green oxalate salt (Sigma-Aldrich, St. Louis, MO), and threonine-phosphopeptide (K-R-pT-I-R-R, pT, New England Peptide LLC, Gardner, MA) were obtained for cell-free assays. All other reagents were obtained from Sigma-Aldrich, unless otherwise noted.

Cell culture

Human pulmonary artery endothelial cells (HPAEC or ECs) were obtained from Lonza (Walkersville, MD) and were cultured as described previously in the manufacturer’s recommended endothelial growth medium-2 (EGM-2). Cells were grown at 37°C in a 5% CO₂ incubator, and passages 6 to 9 were used for experiments. Media was changed one day before experimentation.

Trans-endothelial electrical resistance

Lung EC were grown to confluence in polycarbonate wells containing evaporated gold microelectrodes, and trans-endothelial electrical resistance (TER) monolayer measurements were performed using an electrical cell-substrate impedance sensing system (Applied Biophysics, Troy, NY) as described previously in detail.10 TER values from each microelectrode were pooled as discrete time points and plotted versus time as the mean ± S.E.M.

Cell-free PP2A assay

Recombinant PP2A catalytic subunit was incubated 30 min at 4°C in 4-nitrophenyl-phosphate (pNPP) buffer (50 mM Tris-HCl, pH 7.0, 0.1 mM CaCl₂) plus 5 μM of agonist. Reactions proceeded 10 min at 30°C with intermittent shaking in the presence of pT substrate described above. Reactions were stopped by placing on ice, with aliquots evaluated by malachite green assay relative to freshly prepared phosphate standards and read at 630 nm (Multiskan Spectrum plate reader, Thermo Scientific, Pittsburgh, PA) as detailed in Lek et al.31 PP2A activity is expressed as pmol phosphate/min/μg protein normalized to the methanol vehicle as baseline.

Statistical analysis

For all experiments (n = 3 or more), values are shown as the mean ± SEM, and data were analyzed using standard Student’s t test or two-way ANOVA. Significance in all cases was defined at p < 0.05.

SIP1 receptor docking simulations

The Molecular Operating Environment (MOE) software was used to perform ~300 simulations. Single crystal X-ray data of the SIP1 receptor (PDB file code 3V2Y in MOE) provided coordinates for the SIP1 receptor co-crystallized with the SIP1 antagonist W146 at 2.8 Å resolution. With W146 being localized within the active site of SIP1, the antagonist bound in 3V2Y was substituted with the substrates to be simulated, and the latter were placed into the active site of SIP1 without overlapping any receptor components. The placement phase used in the docking experiments was the default “Induced Fit” method with London dG scoring. The refinement method was set to the Induced Fit receptor model for a more realistic ligand-receptor interaction with GBVI/WSA dG scoring. Each experiment ran 100 ligand poses pre-set within the active site of SIP1, and the 25 energetically most favorable poses were saved. After running all simulations, the results were sorted in ascending order of the energy values of each conformer, and the lowest energy ligand-receptor pose (tightest binding) of each substrate was chosen, displayed in the receptor’s active site, and evaluated for its structural and conformational features. The molecular surface of the receptor’s active site was visualized for structural and steric analysis.

Results

Evaluation of G protein-coupled SIP receptor involvement in (S)- and (R)-FTY720 regioisomer barrier disruption

The (S)- and (R)-FTY720 regioisomers produced potent and dose-dependent barrier disruption in lung ECs as determined by TER, a highly sensitive in vitro measure of permeability. Since SIP1 receptors are critical modulators of barrier regulation by SIP1 and other related compounds, we explored the role of SIP receptors in barrier disruption by the (S)- and (R)-FTY720 regioisomers. Pretreatment of lung ECs with SB649146, an inverse agonist of the SIP1 receptor, attenuated SIP-induced barrier enhancement (Fig. 1) but did not ameliorate FTY720 regioisomer-induced barrier disruption as monitored by TER (Fig. 1). Pretreatment with either JTE-013, a selective SIP2 receptor antagonist, or BML-241, a selective SIP3 receptor antagonist, also did not significantly block TER permeability induced by the EC barrier disruptive (S)- and (R)-FTY720 regioisomers (Fig. 1).

We and others have shown that multiple receptors, such as PAR-1 and APC, are linked to SIP signaling. Activated protein C (APC) regulates EC barrier integrity via endothelial protein C receptor (EPCR). We have previously demonstrated that APC binding to EPCR cleaves the
PAR-1 receptor, thereby activating PAR-1 with subsequent transactivation of S1P1. Exploration of the role of PAR-1 receptors in barrier disruption by the (S)- and (R)-FTY720 regioisomers revealed that a PAR-1 blocking antibody (Fig. 2) attenuated S1P-induced barrier enhancement, due to PAR1-S1P1 transactivation, but failed to alter FTY720 regioisomer-induced barrier disruption. Similarly, preincubation with the G protein-coupled receptor inhibitor PTX produced prominent attenuation S1P-induced barrier enhancement as we previously reported but failed to ameliorate TER permeability induced by the FTY720 regioisomers (Fig. 3). These data demonstrate the absence of typical S1P1 or related sphingolipid receptors (S1P1, S1P2, S1P3, PAR-1, G protein-coupled receptors) involvement in FTY720 regioisomer-mediated barrier signaling, indicating subtle FTY720 structural changes significantly alter binding in ECs.

**FTY720 regioisomer docking simulations with S1P**

The binding behavior of endogenous S1P and synthetic S1P analogues was examined utilizing molecular dynamic docking simulations. As S1P is the endogenous substrate for S1P1, high binding affinity with $K_i$ values from 7.9 to 9.4, this was defined as the standard to which the synthetic analogues and their optimized conformations in the active site of S1P1 were compared with a calculated potential energy of (−351 kcal/mol). The phosphate of the head group is decisive in fixing S1P within the receptor involving residues Lys34 and Tyr110. Additionally, residue Thr109 further stabilizes the head group of S1P within the active site’s cavity of S1P1 (Fig. 4a). The corresponding $K_i$ values for the unphosphorylated (S)- and (R)-FTY720 regioisomers (−1–2 kcal/mol) indicate very weak affinity for S1P1 in the absence of phosphorylation. The binding energies of both regioisomers were similar in value to each other: −1.92 kcal/mol for the (R)-FTY720 regioisomer and −0.81 kcal/mol for the (S)-FTY720 regioisomer, suggesting extremely weak binding affinity, if any, for the unphosphorylated regioisomers to S1P1.
The 3D visualization of the (S)- and (R)-FTY720 regioisomer simulations displays few intermolecular interactions with the receptor’s active site. A “bent” alkyl chain of (R)-FTY720 regioisomer suggests high steric strain contributing to the poor affinity of unphosphorylated (R)-FTY720 regioisomer to S1P1 (Fig. 4b). In the case of (S)-FTY720 regioisomer, only the protonated amine of the substrate exhibits a hydrogen bond to residue Thr109 (Fig. 4c), again supporting the low score achieved when docked in the active site of S1P1. Both simple potential energy analysis and 3D visualization again elucidate that the presence of the negatively-charged phosphate moiety is critical for efficient binding.

Effect of inhibitors of barrier-regulatory pathways on (S)- and (R)-FTY720 regioisomer-mediated barrier disruption

A well-known modulator of EC barrier permeability is the VEGF/VEGFR2 receptor pathway, which has been linked to the S1P1 receptor via crosstalk. We next explored the potential link between VEGF receptors and EC barrier disruption evoked by (S)- and (R)-FTY720 regioisomers. Preincubation of lung EC with a VEGFR2 inhibitor (Fig. 5) resulted in attenuation of S1P-induced barrier enhancement (likely due to VEGFR2-S1P1 crossactivation) but did not ameliorate (S)- or (R)-FTY720 regioisomer-induced barrier disruption.
in the active site of S1P1 with a relative energetic value of -0.81 kcal/mol, which also suggests poor binding to S1P1 in comparison to S1P. The docking simulation of unphosphorylated 3S to S1P1 shows hydrogen binding of the protonated amine of 3S to residue Thr109. In the calculations, the hydroxyl group at C3 further coordinates to a water molecule which in turn hydrogen bonds to residue Lys34. No direct interaction of 3S with any residues except for Thr109 could be observed in the calculations, explaining the poor affinity of the unphosphorylated substrate to S1P1.

S1P: sphingosine-1-phosphate; FTY720: 2-amino-2-(2-[4-octylphenyl])ethyl)-1,3-propanediol.

![Fig. 4. Continued](image)

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S1P: sphingosine-1-phosphate; FTY720: 2-amino-2-(2-[4-octylphenyl(ethyl)]-1,3-propanediol.

![Fig. 5. Effect of VEGFR2 receptor inhibition on (S)- and (R)-FTY720 regioisomer-induced barrier disruption. HPAEC were plated on gold microelectrodes for TER measurements as described in the Methods section. Bar graphs depict pooled TER data from HPAEC pre-treated for 1 h with no inhibitor (grey) or a VEGFR2 kinase IV inhibitor (1 uM, blue), then stimulated with (S)- FTY720 regioisomer (10 uM), (R)-FTY720 regioisomer (10 uM), or S1P (1 uM) as indicated. The data are expressed as change in TER, compared to normalized unstimulated or inhibitor only controls, at 6 h ((S)- and (R)-FTY720 regioisomers) or 10 min (S1P) after agonist stimulation (± S.E.M.). Normalized resistance values over 1 indicate barrier enhancement. Normalized resistance values under 1 indicate barrier disruption. n = 3 independent experiments per condition; *p < 0.01 agonist alone versus unstimulated cells; p < 0.05 agonist with inhibitor pretreatment versus agonist alone.

S1P: sphingosine-1-phosphate; FTY720: 2-amino-2-(2-[4-octylphenyl(ethyl)]-1,3-propanediol.

Pretreatment with various inhibitors of EC barrier-regulatory signaling pathways such as MAPK, NFκB, PI3K, PKC, or Rho-kinase (Fig. 6) failed to attenuate (S)- and (R)-FTY720 regioisomers-evoked permeability. Based on reports of FTY720 effects on phosphatase 2A activity,50–52 we next evaluated (S)- and (R)-FTY720 regioisomer-induced PP2A activity responses. Both FTY720 regioisomers were observed to stimulate PP2A activity (Fig. 7a); however, neither treatment with calyculin A (PP1/2A-C inhibitor) or okadaic acid (PP2A inhibitor) (Fig. 7b) affected the rapid (S)- and (R)-FTY720 regioisomers-induced barrier disruption in lung ECs as monitored by TER. Thus, these studies, designed to mechanistically examine FTY720 regioisomer-mediated barrier disruption, failed to resolve the mechanism of FTY720 regioisomer-induced loss of EC barrier integrity.

**Discussion**

We have attempted to further elucidate the role of FTY720 regioisomers on lung injury, specifically on in vitro pulmonary vascular barrier function and related signaling. Pulmonary vascular leak is a vital therapeutic target in acute inflammatory diseases such as ARDS, a condition afflicting an estimated 400,000 people annually with morbidity rates exceeding 30%. To date, there are no effective treatments that target the underlying pulmonary vascular leak that characterizes this syndrome. In prior studies, we identified S1P1,17,18 and the related pharmacologic agent, FTY720,9,17,53 as potent pulmonary vascular barrier-enhancing agents both in vitro and in vivo. However, as a therapy, S1P suffers from the potential to produce adverse effects, including cardiac toxicity, pulmonary edema at higher doses, and airway hyperresponsiveness.8,20,22–24 Similarly, FTY720 has the potential to induce bradycardia, immune-suppression, and increased vascular leak at higher doses.8,24–26,54 With preclinical models confirming detrimental effects on vascular permeability at higher concentrations,11,27–29 multiple groups have synthesized derivatives of FTY720 in order to identify possible antiangiogenic agents55 that exhibit pro-apoptotic effects,56 S1P receptor affinity, and lymphopenia induction.16,24,57–62 In contrast, we have focused on their value as potential barrier-regulatory therapeutic agents.5,11,19,63 Given the potential therapeutic limitations of S1P and FTY720 in patients with ARDS, this study looked to further characterize FTY720 regioisomers for insight into vascular leak and to subsequently better understand the mechanisms by which novel class of agents can be created to better modulate permeability.

Unlike S1P and FTY720, the (S)- and (R)-FTY720 regioisomers produce potent and dose-dependent barrier disruption in lung ECs8 via poorly understood atypical signaling cascades that do not result in actin stress fiber formation, MLC phosphorylation, ERK phosphorylation, or intracellular calcium release.8 Our studies demonstrate that FTY720 regioisomer-mediated permeability in lung ECs is not altered by attenuation of Gi-coupled receptors, S1P1, S1P2, or S1P3 (Figs 1 and 3), findings supported by S1P1 docking simulations (Fig. 4). Inhibition of the PAR-1 receptor (Fig. 2) or the VEGF receptor (Fig. 5) also did not alter FTY720 regioisomer-induced barrier disruption. Key cytoskeletal regulatory signaling pathways involved in lung EC barrier disruption were not activated during FTY720 regioisomer permeability including Rho-kinase, MAPK, NFκB, or PI3K pathways (Fig. 6). While pretreatment with PP2A inhibitors (Fig. 7b) did not affect rapid FTY720 regioisomers permeability in lung ECs, the (S)- and (R)-FTY720...
Fig. 6. Involvement of various EC barrier-regulatory signaling pathways in (S)- and (R)-FTY720 regioisomer-induced barrier disruption. HPAEC were plated on gold microelectrodes for TER measurements as described in the Methods section. Bar graphs depict pooled TER data from HPAEC pre-treated for 1 h with no inhibitor (grey), PD98059 (MAPK inhibitor, 25 μM, red), CAY10512 (NFκB inhibitor, 4 μM, orange), LY294002 (PI3K inhibitor, 25 μM, green), Go 6983 (PKC α, β, γ, δ, ζ inhibitor, 1 μM, blue), or Y-27632 (Rho-kinase inhibitor, 10 μM, purple), then stimulated with (S)-FTY720 regioisomer (10 μM), (R)-FTY720 regioisomer (10 μM), or S1P (1 μM) as indicated. The data are expressed as change in TER, compared to normalized unstimulated or inhibitor only controls, at 6 h ((S)- and (R)-FTY720 regioisomers) or 10 min (S1P) after agonist stimulation (± SEM). Normalized resistance values over 1 indicate EC barrier enhancement. Normalized resistance values under 1 indicate EC barrier disruption. n = 3–5 independent experiments per condition; *p < 0.01 agonist alone versus unstimulated cells; p < 0.01 agonist with inhibitor pretreatment versus agonist alone.

S1P: sphingosine-1-phosphate; FTY720: 2-amino-2-(2-[4-octylphenyl]ethyl)-1,3-propanediol.

Fig. 7. Effect of (S)- and (R)-FTY720 regioisomers on PP2A activity and induced barrier disruption. (a) Bar graphs depict pooled PP2A activity data quantified as the amount of free PO₄ cleaved from pT substrate relative to a standard curve for methanol vehicle (white), ethanol vehicle (grey), FTY720 (5 μM) (green), (S)-FTY720 regioisomer (5 μM) (blue), or (R)-FTY720 regioisomer (5 μM) (purple) (±S.D.). n = 3 independent experiments per condition; *p < 0.05 agonist versus ethanol vehicle alone, p < 0.05 agonist versus methanol vehicle alone. (b) HPAEC were plated on gold microelectrodes for TER measurements as described in the Methods section. Bar graphs depict pooled TER data from HPAEC pre-treated for 1 h with no inhibitor (grey), CAL inhibitor (calyculin A, PPI/2A-C inhibitor, 2 nM, green), or OKA inhibitor (okadaic acid, PP2A inhibitor, 50 nM, blue), then stimulated with (S)-FTY720 regioisomer (10 μM), (R)-FTY720 regioisomer (10 μM), or S1P (1 μM) as indicated. The data are expressed as change in TER, compared to normalized unstimulated or inhibitor only controls, at 4 h ((S)- and (R)-FTY720 regioisomers) or 10 min (S1P) after agonist stimulation (±S.E.M.). Normalized resistance values over 1 indicate EC barrier enhancement. Normalized resistance values under 1 indicate EC barrier disruption. n = 3 independent experiments per condition; *p < 0.01 agonist alone versus unstimulated cells. S1P: sphingosine-1-phosphate; FTY720: 2-amino-2-(2-[4-octylphenyl]ethyl)-1,3-propanediol.
regioisomers significantly enhanced PP2A activity (Fig. 7a) as previously reported for Parkinson’s disease models using MN9D dopaminergic cells.54–66

In summary, our results demonstrate that subtle FTY720 structural changes significantly alter EC barrier-regulatory properties, similar to our previous reports.19 Despite structural similarity to the parent FTY720 compound, the unphosphorylated (S)- and (R)-FTY720 regioisomers increase EC vascular permeability via an unknown rapid, non-S1P receptor-mediated mechanism that does not involve classic cytoskeletal regulators but whose rapidity suggests a receptor-mediated effect. Given the evolutionary association, sphingolipid receptors, cannabinoid, and other G protein-coupled receptors may be considered as novel (S)- and (R)-FTY720 regioisomer receptors and studies to explore these possibilities are underway. In addition to ligating a potentially novel class of receptors, FTY720 regioisomers are highly hydrophobic, indicating that the highly hydrophobic tail may penetrate the cell membrane without a requirement for receptor binding, to induce rapid increases in EC permeability. Our results with PP2A activity are consistent with this possibility, although we failed to identify evidence of PP2A involvement or cytoskeletal signaling. Thus, our mechanistic insights into FTY720-mediated barrier disruptive mechanisms remain limited. Minor structural alterations in S1P/FTY720 should be evaluated for their impact on vascular permeability regulation while designing novel agents to treat inflammatory disorders such as ARDS.

**Highlights**

- There is an unmet medical need for novel therapies in ARDS
- This study examines the effects of FTY720 regioisomers on vascular permeability
- FTY720 regioisomers do not utilize S1P receptor signaling pathways to alter vascular permeability
- These mechanistic insights may assist in the development of novel ARDS therapeutic strategies.

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**Conflict of interest**

Joe G.N. Garcia, MD is the founder, CEO, and majority shareholder of Aqualung Therapeutics, Corp., which does not have any relevant conflicts of interest. All other authors have no relevant conflicts of interest.

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**ORCID iD**

Joe G. N. Garcia https://orcid.org/0000-0002-6934-0420

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