FTY720 Phosphate Activates Sphingosine-1-Phosphate Receptor 2 and Selectively Couples to \( \text{Ga}^{12/13} \)/Rho/ROCK to Induce Myofibroblast Contraction\[\text{suppl}\]

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ABSTRACT

FTY720 phosphate (FTY720-P; 2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol, monodihydrogen phosphate ester) is a nonselective sphingosine-1-phosphate (S1P) receptor agonist thought to be devoid of activity at the S1P\(_2\) receptor subtype. However, we have recently shown that FTY720-P displays significant S1P2 receptor agonist activity in recombinant cells and fibroblasts expressing endogenous S1P2 receptors. To elucidate the S1P2-dependent signaling pathways that were activated by FTY720-P, we employed second messenger assays and impedance-based assays in combination with pharmacological and small interfering RNA–based pathway inhibition in recombinant Chinese hamster ovary (CHO)–S1P2 cells as well as human lung myofibroblasts generated in vitro. In CHO-S1P2 cells, FTY720-P did not modulate cAMP or calcium levels. However, reporter-gene assays, impedance-based assays with a selective Rho-associated kinase (ROCK) inhibitor, \( \text{Ga}^{12/13} \) knockdown and activated ROCK-pull-down assays demonstrated that FTY720-P potently activated \( \text{Ga}^{12/13} \)/Rho/ROCK signaling. S1P similarly activated \( \text{Ga}^{12/13} \)/Rho/ROCK signaling via S1P2 receptors, whereas the two selective S1P1 receptor agonists \([\text{Z,Z}]-5-(3-	ext{chloro}-4-[2\text{R}-2,3-	ext{dihydroxy-proproxyl]}-	ext{benzylidene})-2-	ext{propylinino}-3-	ext{o-tolyl-thiazolidin}-4-	ext{one (ponesimond)} and \([5-4-	ext{phenyl}-5-(	ext{trifluoromethyl})\text{thiophen}-2-y]3-	ext{[trifluoromethyl]phenyl}]1,2,4-	ext{oxadiazole (SEW2871)} were inactive. In lung myofibroblasts, which mainly expressed the S1P2 receptor subtype, we showed that FTY720-P selectively activated the \( \text{Ga}^{12/13} \)/Rho/ROCK pathway via the S1P2 receptor. Moreover, the activation of the \( \text{Ga}^{12/13} \)/Rho/ROCK pathway in myofibroblasts by FTY720-P caused potent myofibroblast contraction similar to that induced by the natural ligand S1P. Thus, complementing second messenger assays with unbiased label-free assays or phenotypic assays in native expression systems can uncover activation of additional pathways, such as \( \text{Ga}^{12/13} \)/Rho/ROCK signaling.

Introduction

Sphingosine-1-phosphate (S1P) receptors constitute a family of five related G protein–coupled receptors (GPCRs) named S1P1,2,3,4,5, receptors, which are activated by the natural ligand S1P and are involved in pleiotropic physiologic processes (Brinkmann, 2007). Upon agonist binding, GPCRs transmit extracellular signals to the inside of cells through activation of one or several intracellular heterotrimeric G protein subtypes to modulate cAMP levels (\( \text{Ga}^q \) or \( \text{Ga}^i \) proteins), increase calcium levels (\( \text{Ga}^i \) proteins), or activate RhoGTPase/Rho-associated kinases (ROCK1/2) (\( \text{Ga}^{12/13} \) proteins), the latter resulting in cellular responses that include gene transcription, rearrangement of the actin cytoskeleton, and cellular shape changes (Siehler, 2009; Kozasa et al., 2011; Zhang and Xie, 2012).

Synthetic S1P receptor modulators constitute a new class of drugs for the treatment of autoimmune diseases, and the first nonselective S1P receptor modulator fingolimod (FTY720/Gilenya; Novartis, Basel, Switzerland) was approved for the treatment of relapsing-remitting multiple sclerosis (Brinkmann et al., 2010). Recently, it was shown that the natural ligand S1P and the active phosphorylated metabolite of fingolimod [FTY720 phosphate (FTY720-P), 2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol, monodihydrogen phosphate ester] can induce fibrotic responses in isolated fibroblasts of different tissues and fingolimod treatment caused fibrotic lung changes in rats and monkeys (Urata et al., 2005; Keller et al., 2007; Gellings Lowe et al., 2009; Gilenya package insert, 2010; Sobel et al., 2013). Most publications

ABBREVIATIONS: CHO, Chinese hamster ovary; CRC, concentration–response curve; FAF-BSA, fatty acid–free bovine serum albumin; FLIPR, fluorescence imaging plate reader; FTY720-P, FTY720 phosphate, 2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol, monodihydrogen phosphate ester; GPCR, G protein–coupled receptors; SEW2871, 5-[4-phenyl-5-(trifluoromethyl)thiophen-2-y]3-[trifluoromethyl]phenyl]1,2,4-oxadiazole; siRNA, small interfering RNA; SRF-RE, serum response factor response element; U73122, 1-[6-[((17\_\beta)-3-methoxyestra-1,3,5(10)-tri-en-17-yl]amino)hexyl]-1H-pyrrole-2,5-dione.
based on GTPγS binding assays and second messenger assays describe FTY720-P as a nonselective S1P1,3,4,5 receptor agonist that lacks activity at the S1P2 receptor subtype (Brinkmann et al., 2002; Brinkmann, 2007). However, when we studied the induction of profibrotic responses in normal human lung fibroblasts (NHLFs) using S1P receptor agonists with different receptor subtype selectivity profiles, we uncovered S1P2 receptor activation by FTY720-P using impedance-based technology and demonstrated that S1P2 receptor activation in fibroblasts contributed to the induction of profibrotic responses (Sobel et al., 2013). In contrast, we found that (Z, Z)-5-[(3-chloro-4-{(2R, 2,3-dihydroxy-propoxy)-benzylidene})-2-propilimino-3-o-tolyl-thiazolidin-4-one (ponesimod) and 5-{[4-phenyl-5-(trifluoromethyl)thiophen-2-yl]-3-[trifluoromethyl]phenyl]1,2,4-oxadiazole (SEW2871), two selective S1P1 receptor modulators, lacked S1P2 receptor activity in GTPγS assays as well as S1P2 receptor impedance assays and did not induce profibrotic responses through S1P2 receptor activation (Bolli et al., 2010; Piali et al., 2011; Sobel et al., 2013). These findings raised the possibility that S1P2 receptor activation by FTY720-P was not captured by classic GPCR assays that measure the second messengers cAMP and calcium, but its detection required unbiased label-free assays instead. Label-free techniques, such as dynamic mass redistribution or impedance measurements, which allow for noninvasive, real-time analysis of receptor activation within cell monolayers, are established technologies to analyze GPCR signaling in an unbiased fashion (Nayler et al., 2010; Schroder et al., 2010) and are discussed as a complementation to classic GPCR assays, which are based on quantification of distinct intracellular second messengers. Impedance-based assays, in particular, are sensitive methods to accurately quantify even minute changes in cell shape.

In this study, we further characterized the FTY720-P–induced S1P2 receptor activity by measuring the activity of FTY720-P and selected S1P receptor agonists in classic second messenger and impedance-based assays. We show that FTY720-P did not modulate cAMP or calcium pathways in recombinant Chinese hamster ovary (CHO)–S1P2 cells, but triggered Ga13/Rho/ROCK activation, whereas the selective S1P1 receptor agonists ponesimod and SEW2871 were inactive. We then extended our studies to primary human lung myofibroblasts, which mainly express the S1P2 receptor subtype (Sobel et al., 2013) and demonstrate that FTY720-P selectively activated the Ga13/Rho/ROCK pathway in the absence of detectable cAMP or calcium modulation. The S1P2 receptor–mediated activation of the Ga13/Rho/ROCK pathway by FTY720-P led to pronounced contractile responses of primary human myofibroblasts in collagen-gel contraction assays. Thus, label-free technologies and native cells can be used to improve the pharmacological profiling of GPCR modulators and, in the case of FTY720-P, may uncover potentially safety-related activities of compounds, which would have been overlooked using only classic GPCR assays.

Materials and Methods

The following reagents were used: S1P (Enzo Life Sciences, Lausen, Switzerland); 1-{[3,3-dimethyl-4-(2-methylthethyl)-1H-pyrazolo[3,4-b]pyridin-6-yl]-4(3,5-dichloro-4-pyridinyl)-semicarbazide (JTE-013), SEW2871, and N-[3-{[2-(4-amino-1,2,5-oxadiazol-3-yl)1-ethyl-1H-imidazo[4,5-c]pyridin-6-yl]oxy}phenyl]-4-[2-(4-morpholinline)ethoxy]benzamide (GSK269962) (Tocris Bioscience, Abingdon, United Kingdom); FTY720-P (Cayman Chemical, Tallinn, Estonia); lysophosphatidic acid (LPA) (Avanti Polar Lipids, Alabaster, AL); and forskolin, serotonin hydrochloride, human calcitonin, thrombin, transforming growth factor β1 (TGF-β1), and 1-[6-{[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino}hexyl]-1H-pyrrole-2,5-dione (U73122) (Sigma-Aldrich, Buches, Switzerland). Ponesimod was synthesized by Actelion Pharmaceuticals Ltd (Allschwil, Switzerland) as described (Bolli et al., 2010).

Cell Culture. CHO-K1 cells that were stably transduced with plasmids encoding for the human S1P2 receptor (CHO-S1P2) or with pcDNA3.1 (CHO–empty vector) were grown in Ham’s F12/10% fetal bovine serum/penicillin (100 U)/streptomycin (1 μg/ml)/geneticin (1 mg/ml) (all from Life Technologies, Zug, Switzerland). Myofibroblasts were generated by incubating NHLFs (female 19 years; Lonza, Verviers, Belgium) in fibroblast growth medium 2 (Lonza), with 1 ng/ml TGF-β1, for 72–96 hours before experiments were performed. Preincubation with inhibitors and agonist stimulation were performed as described below. Controls and all samples were supplemented with equal concentrations of dimethyl sulfate, ethanol, or methanol (vehicles).

Impedance Measurements. CHO cells were seeded at 40,000 cells per well into gelatin-coated E-plates (Roche Applied Science, Rotkreuz, Switzerland). Myofibroblasts were seeded at 10,000 cells per well into E-plates. Both cell types were subjected to continued impedance sampling over the whole experimental period (xCELLigence system; Roche Applied Science). After overnight growth, the medium was exchanged with starvation medium for 1 hour (CHO cells: Ham’s F12/0.1% fatty acid–free bovine serum albumin (FAB-BSA) (Calbiochem, Darmstadt, Germany)/penicillin (100 U)/streptomycin (1 μg/ml)/geneticin (1 mg/ml)) or 24 hours [myofibroblasts: fibroblast basal medium (Lonza)/0.1% FAB-BSA/penicillin (100 U)/streptomycin (1 μg/ml)]. When indicated, cells were preincubated with or without JTE-013 (0.2 or 1 μM), GSK269962 (40 nM), or U73122 (5 μM) for 1–2 hours, followed by stimulation with compounds at various concentrations. For data analysis, impedance raw traces were normalized at the time point of agonist addition and the baseline response (vehicle instead of agonist) was subtracted.

Small Interfering RNA Transfection. CHO-S1P2 cells or myofibroblasts (both 106 cells/well) were seeded into E-plates and 24 hours later were transfected with Lipofectamine RNAiMAX (Life Technologies) following the manufacturer’s protocol. The following siRNAs (Ambion, Austin, TX) were used: silencer select negative control siRNA #4390543 at 33 nM, #222246 for mouse GNA12 at 33 nM (CHO cells; sequence identical to hamster sequence), s8867 for human GNA12 at 33 nM (myofibroblasts), and s20990 for human GNA13 at 100 nM (CHO cells (sequence identical to hamster sequence) and myofibroblasts). After transfection, cells were starved for 48 hours [CHO cells: Ham’s F12/0.1% FAB-BSA/penicillin (100 U)/streptomycin (1 μg/ml)/geneticin (1 mg/ml); myofibroblasts: fibroblast basal medium/0.1% FAB-BSA/penicillin (100 U)/streptomycin (1 μg/ml)] and then stimulated with vehicle or S1P agonists as indicated. All samples were subjected to continued impedance sampling over the whole experimental period as described above. Knockdown of human Ga13 and Ga13 mRNA was controlled by quantitative real-time polymerase chain reaction (qPCR).

Measurement of Intracellular cAMP Levels. CHO-S1P2 cells were seeded at 60,000 cells/well and myofibroblasts were seeded at 40,000 cells/well into 96-well plates and incubated overnight at 37°C in 5% CO2. Then, cells were incubated with 0.5 mM 3-isobutyl-1-methylxanthine for 15 minutes and stimulated with compounds at various concentrations in the absence or presence of 10 μM forskolin for 30 minutes. Cells were lysed, and cAMP levels in the lysates were determined using the cAMP-Screen 96-well cyclic AMP immunoassay system (Life Technologies) following the manufacturer’s protocol.

Intracellular Calcium Release Measurements. CHO-S1P2 cells and myofibroblasts were seeded at 10,000 cells/well into 384-well black clear-bottom plates (Greiner, Frickenhausen, Germany)
and incubated overnight at 37°C in 5% CO₂. Growth medium was then exchanged by dye buffer [Hank’s balanced salt solution (HBSS), 0.1% FAF-BSA, 20 mM HEPES, 0.375 g/l sodium bicarbonate (NaHCO₃)], 5 mM probenecid (Sigma-Aldrich), 3 μM N-[4-(6-[(acetyloxy)ethoxy]-2,7-difluoro-3-oxo-3H-xanthen-9-yl]-2-[[2-(2-[bis[(2-[(acetyloxy)ethoxy]-2-oxoethyl)amino]-5-methylphenoxy]ethoxy)phenyl]-N-[2-(acetyloxy)ethoxy]-2-oxoethyl]-(acetyloxy)methyl ester (fluo-4 AM) (Life Technologies), pH 7.4, and cells were incubated for 1 hour at 37°C in 5% CO₂ followed by 1× washing (HBSS, 0.1% FAF-BSA, 0.375 g/l NaHCO₃, 2.5 mM probenecid, pH 7.4) and equilibration in washing buffer at room temperature for 1 hour. When inhibitors were used, cells were preincubated with or without inhibitors (JTE-013 at 0.5 μM or U73122 at 5 μM) diluted in dilution buffer (HBSS, 0.1% FAF-BSA, 20 mM HEPES, 0.375 g/l NaHCO₃, pH 7.4) for 1 hour. Then, cells were stimulated with agonists diluted in dilution buffer at various concentrations. Calcium release was monitored for 3 minutes with the fluorescent imaging plate reader (FLIPR Tetra; Molecular Devices, Sunnyvale, CA). FLIPR traces were normalized by trace alignment at the last time point before agonist addition (ScreenWorks software; Molecular Devices). Then, relative fluorescence units of the maximum signal per well were exported and presented as concentration-response curves.

**Reporter Gene Transcription Assay.** CHO cells were seeded at 10,000 cells/well into gelatin-coated 96-well plates and incubated overnight at 37°C in 5% CO₂. Then, cells were transfected with pGL4.34[ACt2P/SRF-RE/Hygro] vector plasmid (Promega, Duebendorf, Switzerland) using Lipofectamine LTX (Life Technologies). After 6 hours, medium was exchanged to Ham’s F12/0.5% fetal bovine serum/penicillin (100 U)/streptomycin (1 μg/ml)/geneticin (1 mg/ml). The next day, the medium was exchanged to Ham’s F12 0.1% FAF-BSA/penicillin (100 U)/streptomycin (1 μg/ml)/geneticin (1 mg/ml) before stimulation, with compounds at various concentrations for 6 hours. Luciferase activity was analyzed using the One-Glo luciferase assay system (Promega), and the induction was expressed as percentage increase over control (vehicle treatment).

**Rho-GTP Pull-Down Assay.** The Active Rho Detection Kit (Cell Signaling Technologies, Danvers, MA) was used. Briefly, CHO-S1P₂ cells were seeded at 5 × 10⁶ cells/10-cm dish. Cells were starved after 24 hours (Ham’s F12/0.1% FAF-BSA/penicillin (100 U)/streptomycin (1 μg/ml)/geneticin (1 mg/ml)). After an additional 24 hours, cells were stimulated with vehicle, S1PR agonists, or LPA for 5 minutes. Lysates were then processed following the manufacturer’s protocol.

**Contraction Assay.** The cell contraction assay kit from Cell Biolabs (San Diego, CA) was used. Briefly, one volume of myofibroblast suspension (1 × 10⁶ cells/well) was mixed with four volumes of collagen gel lattice mixture, plated into wells of 48-well plates, and incubated for 1 hour at 37°C according to the manufacturer’s protocol. Then, 0.5 ml of fibroblast growth medium 2/1 ng/ml TGF-β1 was added and gel lattices were incubated for 24 hours. The medium was exchanged to fibroblast basal medium (Lonza)/0.1% FAF-BSA (Calbiochem)/penicillin (100 U)/streptomycin (1 μg/ml) for an additional 24 hours. When inhibitors were used, gel lattices were preincubated with or without JTE-013 (1 μM) or GSK269962 (80 nM) for 2 hours. Gel lattices were then released from the sides of wells and stimulated with agonists at the indicated concentrations. Pictures were taken before and 30 minutes after agonist addition, and the area of the gel lattices was measured using Multi Gauge Software V3 (Fujiﬁlm, Düsseldorf, Germany). The cell contraction was determined by dividing the gel lattice area 30 minutes after stimulation by the initial area, and contraction was expressed as the relative gel size compared with correspondingly pretreated (vehicle or antagonists without agonists) controls at 30 minutes.
Statistics and EC₅₀ Calculation. For statistical analysis, the one-way analysis of variance with Dunnett’s post hoc test was performed. When the P value was <0.05, the results were considered significant (GraphPad 6 software; GraphPad Software, San Diego, CA). EC₅₀ values were calculated using the proprietary software IC₅₀ Witch and the curve intrinsic maximum or minimum as assigned maximum or minimum.

Results
Impedance-Based Analysis of S₁P₂ Receptor Signaling in CHO-S₁P₂ Cells Reveals Activation of S₁P₂ Receptors by FTY720-P. To elucidate the FTY720-P/S₁P₂ receptor coupling in CHO-S₁P₂ cells, we performed concentration response experiments using an impedance-based assay system. As shown exemplarily for 500 nM FTY720-P in Fig. 1A, a rapid impedance decrease with a maximal reduction at ~1.5 minutes was observed, which was followed by a gradual increase over baseline (solid line). This response was completely blocked by the S₁P₂ receptor antagonist JTE-013 (dashed line), suggesting that the observed response was S₁P₂ receptor mediated. The S₁P₂ receptor dependency of the FTY720-P–induced impedance response was further substantiated by the absence of impedance responses in FTY720-P–stimulated CHO-K1 empty vector cells (Fig. 1B). To compare the S₁P₂ receptor–mediated responses of FTY720-P, S₁P, ponesimod, or SEW2871, concentration–response curves (CRCs) were generated from impedance values at the time point of maximal reduction (1.5 minutes) (Fig. 1C). S₁P and FTY720-P induced a concentration dependent, and for FTY720-P, saturable decrease of impedance (EC₅₀ = 35 nM; n = 5), whereas the selective S₁P₁ receptor

![Graph showing concentration-response curves for different agonists](image)

Fig. 2. Analysis of cAMP and calcium modulation by S₁P receptor agonists in CHO-S₁P₂ cells. (A) CHO-S₁P₂ cells were stimulated with FTY720-P (FTY-P), S₁P, or ponesimod (pones.) (all 1.6–1000 nM) or calcitonin (0.001–1000 nM) for 30 minutes, and cAMP concentrations were measured. (B) CHO-S₁P₂ cells were stimulated with FTY720-P, S₁P, or ponesimod (all 1.6–1000 nM) or serotonin (0.01–1000 nM) in the presence of forskolin (10 μM) for 30 minutes, and cAMP concentrations were measured. (C) CHO-S₁P₂ cells were stimulated with FTY720-P, S₁P, or ponesimod (all 1.6–5000 nM) or thrombin (0.0016–5 units/ml), and the calcium release was recorded. CRCs were then generated showing the peak fluorescence (relative fluorescence units [RFU]). (D) CHO-S₁P₂ cells were preincubated with vehicle, JTE-013 (0.5 μM), or U73122 (5 μM) for 1 hour before stimulation with S₁P (1.6–5000 nM), and calcium release was recorded. CRCs were then generated showing the peak fluorescence (relative fluorescence units). Data in (A–D) show representative experiments with duplicates ± S.D. Experiments were independently repeated three times. anta., antagonist; veh., vehicle.
agonists ponesimod and SEW2871 were both inactive. These data confirm that FTY720-P is a potent and efficacious S1P\textsubscript{2} receptor agonist in CHO-S1P\textsubscript{2} cells using label-free impedance-based assays. We thus sought to identify the intracellular signaling pathways that led to cellular shape changes.

**FTY720-P Does Not Modulate Intracellular cAMP or Calcium Levels in CHO-S1P\textsubscript{2} Cells.** We analyzed FTY720-P, S1P, and ponesimod with respect to the modulation of the classic GPCR second messengers cAMP and calcium in CHO-S1P\textsubscript{2} cells (Zhang and Xie, 2012). To investigate potential G\textsubscript{\alpha}\textsubscript{cs}-mediated adenylate cyclase activation, intracellular cAMP levels were quantified 30 minutes after stimulation with a dilution series of the three different S1P receptor agonists (FTY720-P, ponesimod, and S1P) or the positive control calcitonin (George et al., 1997). Calcitonin induced a potent (EC\textsubscript{50} \sim 3 nM) and robust increase in cAMP levels, reaching a maximum of 35 pmol/well cAMP at 1000 nM due to activation of the endogenous calcitonin receptor. In contrast, all three S1P receptor agonists were inactive in this assay up to the highest tested concentration (1000 nM) (Fig. 2A). To study potential G\textsubscript{\alpha}\textsubscript{i}-mediated adenylate cyclase inhibition, we stimulated the CHO-S1P\textsubscript{2} cells for 30 minutes with a dilution series of FTY720-P, S1P, ponesimod, or the positive control serotonin (George et al., 1997) in the presence of forskolin, which stimulates adenylate cyclase activity. Serotonin, which is

Fig. 3. Probing for G\textsubscript{\alpha}\textsubscript{12/13}/Rho/ROCK activation induced by S1P receptor agonists in CHO-S1P\textsubscript{2} cells using impedance-based assays. (A and B) CHO-S1P\textsubscript{2} cells were preincubated with vehicle, GSK269962 (40 nM), or U73122 (5 \textmu M) for 2 hours before stimulation with FTY720-P or S1P (0.5–5000 nM), and impedance responses were followed. CRCs of FTY720-P (A) and S1P (B) in the absence or presence of the inhibitors were then generated with impedance values at the time point of maximal reduction of impedance (\sim 1.5 minutes). (C) CHO-S1P\textsubscript{2} cells were transfected with negative control siRNA, G\textsubscript{\alpha}12 siRNA, G\textsubscript{\alpha}13 siRNA, or the combination of G\textsubscript{\alpha}12/G\textsubscript{\alpha}13 siRNAs before cells were stimulated 48 hours later with 500 nM FTY720-P (FTY-P) or 500 nM S1P. Impedance values at the time of maximal reduction were then converted into bar graphs. (D) CHO-S1P\textsubscript{2} cells were stimulated with vehicle (co), FTY720-P, S1P, ponesimod (pones.), or LPA (all 5 \textmu M) for 5 minutes, and active GTP-Rho pull-down assays and immunoblotting for Rho were performed. Upper panel: active GTP-bound Rho after pull down; lower panel: total Rho in cell lysates. Data in (A and B) show mean \pm S.E.M. of three independent experiments. Data in (C) show a representative experiment (mean of duplicates \pm S.D.) (n = 2). Data in (D) show a representative experiment (n = 2).
known to activate endogenous 5-hydroxytryptamine 1 receptors in CHO-K1 cells, potently reduced the forskolin-induced cAMP levels almost to baseline (EC$_{50}$ ~10 nM), whereas the S1P receptor agonists did not significantly decrease the forskolin-induced cAMP accumulation up to the highest tested concentration of 1000 nM (Fig. 2B). Thus, the S1P receptor agonists did not modulate cAMP levels.

We then analyzed the potential of the S1P receptor agonists to induce intracellular calcium release via S1P2 receptors. To this end, CHO-S1P2 cells were stimulated with increasing concentrations of FTY720-P, S1P, ponesimod, or the positive control thrombin (Li et al., 2006). Intracellular calcium changes were monitored using FLIPR. Peak fluorescence values were converted into CRC, which are depicted in Fig. 2C. FTY720-P and ponesimod were inactive at all tested concentrations, whereas thrombin and S1P induced a concentration-dependent and saturable calcium release, with an EC$_{50}$ of 0.1 U/ml (range, 0.04–0.14 U/ml) and 46 nM (range, 22.5–112 nM), respectively ($n = 3$). To further investigate the origin of the S1P-induced calcium, we performed calcium release measurements in the presence of the phospholipase C inhibitor U73122 or the S1P2 receptor antagonist JTE-013. As shown in Fig. 2D, U73122 as well as JTE-013 fully blocked the S1P-induced calcium release, demonstrating that the intracellular calcium response was triggered through S1P2 receptor–mediated activation of the phospholipase C pathway.

In summary, in contrast to label-free impedance assays, classic second messenger assays, such as those measuring cAMP modulation and intracellular calcium release, did not capture FTY720-P–induced S1P2 receptor activation, whereas they did capture the calcium response to the natural ligand S1P.

**Impedance-Based Assays with a ROCK Inhibitor and G$_{12/13}$ siRNA in CHO-S1P2 Cells Reveal S1P2 Receptor–Mediated Activation of G$_{12/13}$/Rho/ROCK by FTY720-P.** After excluding a modulation of cAMP and calcium levels by FTY720-P in CHO-S1P2 cells with second messenger assays, we investigated the impedance responses elicited by FTY720-P in the presence of the ROCK inhibitor GSK269962 to block potential G$_{12/13}$-mediated Rho/ROCK activation. The validation of GSK269962 as a selective and specific inhibitor of Rho/ROCK signaling in impedance assays using agonist-receptor pairs with a well established subtype coupling is shown in Supplemental Fig. S1. We stimulated CHO-S1P2 cells with FTY720-P and S1P (both 0.5–5000 nM) in the absence (solid lines) or presence (finely dashed lines) of GSK269962, and generated CRCs at the time of maximal impedance reduction (~1.5 minutes) (Fig. 3, A and B). In the absence of GSK269962, FTY720-P and S1P induced a concentration-dependent impedance decrease starting at 5 nM. Preincubation with GSK269962 fully blocked the FTY720-P- and S1P–induced decrease of impedance, demonstrating Rho/ROCK pathway activation by FTY720-P and S1P. To analyze whether, in the case of S1P, the decrease of impedance was at least in part calcium dependent, we preincubated the cells with the phospholipase C inhibitor U73122 (dashed lines). However, this pretreatment did not alter the S1P-induced impedance response, suggesting that the impedance changes did not reflect the S1P-induced calcium response in these cells. The addition of the inhibitors alone had no (U73122) or only a minor (GSK269962) effect on cellular impedance (Supplemental Fig. S2).

These results suggested that FTY720-P–activated S1P2 receptors are coupled to Rho/ROCK signaling, most probably via G$_{12/13}$ activation. To directly probe for the role of G$_{12/13}$ in the signaling response of S1P2 receptors stimulated with FTY720-P or S1P, we performed siRNA-mediated knockdown of G$_{12}$ and/or G$_{13}$ proteins followed by stimulation with FTY720-P (500 nM) or S1P (500 nM) and measurement of impedance changes. The impedance values at the time point of maximal reduction were converted into bar graphs (Fig. 3C). Knockdown of G$_{12}$ led to a partial inhibition of the FTY720-P–induced impedance response, whereas knockdown of G$_{13}$ or simultaneous knockdown of G$_{12}$ and G$_{13}$ caused a complete loss of response to FTY720-P compared with cells treated with negative control siRNA. Similar effects were observed when S1P was used as a stimulus. To directly monitor Rho activation, we treated CHO-S1P2 cells with FTY720-P, S1P, ponesimod, LPA (each 5 μM), or vehicle and then analyzed Rho activation using Rho-GTP pull-down...
assays followed by anti-Rho immunoblotting. As shown in Fig. 3D (upper panel: isolated activated Rho; lower panel: total Rho in lysate), FTY720-P, S1P, and LPA induced substantial Rho activation, whereas ponesimod did not.

Taken together, these results demonstrate the activation of the G_{12/13}/Rho/ROCK pathway by FTY720-P through S1P_{2} receptors. Furthermore, impedance experiments suggested a selective activation of the G_{12/13}/Rho/ROCK pathway by FTY720-P. To confirm the activation of the G_{12/13}/Rho/ROCK pathway by FTY720-P and S1P in CHO-S1P_{2} cells, we employed a G_{12/13}/Rho/ROCK pathway–specific reporter gene assay measuring transcriptional activation of firefly luciferase under control of the serum response factor response element (SRF-RE) (Mao et al., 1998; Liu and Wu, 2004; Cheng et al., 2010; Zhang and Xie, 2012). After transient transfection of the CHO-S1P_{2} cells with the reporter gene construct, cells were stimulated with different concentrations of FTY720-P, S1P, ponesimod, positive control LPA, or vehicle (Fig. 4A). FTY720-P increased the SRF-RE transcriptional response (EC_{50} = 96.2 nM; n = 5), reaching a plateau at 1000 nM, at which FTY720-P induced an 80% increase in luciferase activity over baseline. Similarly, S1P induced a concentration-dependent and saturable activation of SRF-RE transcription (EC_{50} = 54.9 nM; n = 3) and a maximal response of 165% over control at 1000 and 5000 nM. Ponesimod was inactive at all tested concentrations. LPA, a known activator of Rho/ROCK signaling, induced a concentration-dependent transcriptional activation of SRF-RE, reaching 265% increase in luciferase activity over control at 5000 nM. To show that the FTY720-P– and S1P–induced activation of SRF-RE was S1P_{2} receptor mediated, we stimulated CHO-K1 empty vector cells with the S1P receptor agonists (all 5000 nM), LPA (1000 nM), or vehicle (Fig. 4B). LPA induced a 150% increase of activity, whereas FTY720-P, S1P, and ponesimod were inactive, demonstrating that the responses observed for FTY720-P and S1P were mediated through human S1P_{2} receptors. Therefore, as already suggested by the impedance assays, FTY720-P activity at S1P_{2} receptors is not captured by classic second messenger assays but can be measured using label-free impedance assays or dedicated reporter gene assays probing G_{12/13}/Rho/ROCK activation. Taken together, in CHO-S1P_{2} cells, FTY720-P was a biased agonist selectively activating the Rho/ROCK pathway in the absence of second messenger release, thereby differing from the natural ligand S1P, which also activated calcium signaling.

**FTY720-P Activates S1P_{2} Receptor–Mediated G_{12/13}/Rho/ROCK Signaling and Cell Contraction in Human Lung Myofibroblasts.** We next investigated the coupling of FTY720-P–stimulated S1P_{2} receptors in primary human myofibroblasts and the phenotypic consequences. We had previously shown that NHLF–derived myofibroblasts mainly expressed the S1P_{2} receptor subtype and that these cells responded to FTY720-P and S1P via S1P_{2} receptors in impedance-based assays (Sobel et al., 2013). Here, we first characterized the modulation of intracellular cAMP and calcium levels in NHLF–derived myofibroblasts using second messenger assays. As shown in Fig. 5A, analysis of cAMP modulation in the absence or presence of forskolin did not reveal any significant responses for the three S1P receptor agonists, whereas iloprost, an agonist of the endogenous prostacyclin receptor, induced a strong cAMP increase. Furthermore, neither FTY720-P nor S1P or ponesimod induced any measurable calcium release in these cells, in contrast to LPA, which is a known activator of both calcium and Rho signaling pathways in myofibroblasts (Fischer et al., 1998; Choi et al., 2010) (Fig. 5B). Thus, none of the three investigated S1P receptor agonists modulated cAMP or calcium levels in these cells. Next, we stimulated myofibroblasts with different concentrations of FTY720-P, S1P, or
Fig. 6. Analysis of Rho/ROCK activation by S1P receptor agonists in NHLF-derived myofibroblasts using impedance-based assays. NHLF-derived myofibroblasts were stimulated with FTY720-P, S1P, or ponesimod (all 0.5–5000 nM) in the absence or presence of GSK269962 (GSK; 40 nM) or JTE-013 (0.2 μM), and impedance responses were followed for 6 hours. (A, C, and E) Impedance traces for S1P receptor agonists at 500 nM (S1P) or 5000 nM (FTY720-P or ponesimod) concentrations. (B, D, and F) Concentration-response curves for the same agonists.
ponesimod in the absence or presence of the S1P$_2$ receptor antagonist JTE-013 or the ROCK inhibitor GSK269962 and followed impedance changes for 6 hours. The two inhibitors by themselves had no (JTE-013) or minor (GSK269962) effects on impedance values (data not shown). Figure 6A depicts the response to 5000 nM FTY720-P in the absence (solid line) or presence of the S1P$_2$ receptor antagonist JTE-013 (dashed line) or the ROCK inhibitor GSK269962 (finely dashed line). The CRCs that were generated from impedance values obtained at the 90-minute time point are shown in Fig. 6B. Preincubation with JTE-013 completely blocked the FTY720-P–induced long-lasting decrease of impedance. Moreover, preincubation with GSK269962 totally abolished the FTY720-P–induced impedance decrease, unmasking a considerable concentration-dependent impedance increase (Fig. 6, A and B). Comparable results were obtained with S1P (Fig. 6, C and D). In the presence of JTE-013, the CRC of S1P was shifted 10-fold to lower potency, demonstrating that S1P$_2$ receptors were mediating the observed impedance decrease. In the presence of GSK269962, the impedance decrease was completely abolished, leaving a residual impedance increase demonstrating that Rho/ROCK pathway activation via the S1P$_2$ receptor was mediating the impedance decrease. The residual impedance response to FTY720-P and S1P could be attributed to S1P receptor subtype activation (Supplemental Fig. S3A) that was previously masked by a dominant S1P$_2$-Rho/ROCK signaling. Ponesimod did not induce significant impedance changes, and no major effects of the S1P$_2$ receptor antagonist were observed (Fig. 6E). The ROCK inhibitor pretreatment uncovered a modest impedance increase upon ponesimod addition (Fig. 6F), which could be attributed to a residual activation of S1P receptors (Supplemental Fig. S3B). To analyze for direct involvement of G$_{12/13}$ proteins in the response to FTY720-P or S1P, we performed siRNA-mediated knockdown of G$_{12}$ and/or G$_{13}$ in myofibroblasts, followed by stimulation with FTY720-P (5000 nM) or S1P (500 nM) and impedance measurements. As shown in Fig. 7A, knockdown of G$_{13}$ mRNA (62% by qPCR) did not affect FTY720-P–induced impedance changes. In contrast, knockdown of G$_{12}$ mRNA (61% by qPCR) or simultaneous knockdown of G$_{12}$ and G$_{13}$ led to inhibition of the FTY720-P–induced impedance response by half. Very similar results were obtained using S1P as a stimulus (Fig. 7B). Taken together, these data suggest that the response downstream of the S1P$_2$ receptor is mediated by G$_{12/13}$/Rho/ROCK signaling in primary myofibroblasts in the absence of detectable second messenger signaling.

Cell contraction is a major cellular effect of G$_{12/13}$/Rho/ROCK activation, and myofibroblast contraction is of physiologic and pathophysiologic relevance (Riento and Ridley, 2003; Hinz et al., 2012). The prolonged and pronounced decrease of impedance that we observed after stimulation of myofibroblasts with FTY720-P and S1P demonstrated a major cellular shape change upon stimulation, which is possibly attributable to cellular contraction. To directly assess S1P receptor agonist–induced myofibroblast contraction and analyze contributing pathways, we embedded TGF-β1–generated myofibroblasts into collagen gel lattices and stimulated those cells with different concentrations of FTY720-P, S1P, ponesimod, or LPA. Thirty minutes after stimulation, the areas of the agonist-contracted gels were compared with the corresponding vehicle-treated collagen gel. As seen in Fig. 8A, 5000 nM of FTY720-P, S1P, and LPA induced a strong contraction of the gel, whereas the ponesimod-treated gel lattices were comparable in area to the vehicle control. Quantification of relative gel lattice areas treated with various concentrations of compounds revealed a concentration-dependent gel contraction induced by FTY720-P, S1P, and LPA. The maximal contraction at 5000 nM was comparable between all three compounds, causing a ~50% reduction in gel area compared with controls. The EC$_{50}$ values (n = 3) were 239 nM (FTY720-P), 38 nM (S1P), and 88 nM (LPA). In contrast, ponesimod was inactive at all tested concentrations (Fig. 8B). To analyze the signaling pathway involved in FTY720-P– and S1P–induced cell contraction, we performed the cell contraction assay in the absence or presence of the S1P$_2$ receptor antagonist JTE-013 or the ROCK inhibitor GSK269962 and incubated the gel lattices with FTY720-P (5000 nM), S1P (500 nM), ponesimod (5000 nM), or LPA (5000 nM) (Fig. 8C). The FTY720-P– and S1P–induced gel
contraction was strongly or completely inhibited by JTE-013 or GSK269962, respectively. Ponesimod was inactive and, thus, preincubation with inhibitors did not show any effect. The LPA-induced gel contraction was not inhibited by JTE-013, but was completely prevented by GSK269962. Thus, both FTY720-P and S1P induced myofibroblast contraction via the S1P2/Gα12/13/Rho/ROCK pathway in the absence of second messenger generation.

**Discussion**

In a previous study, we recently found that FTY720-P activated S1P2 receptors in recombinant CHO-S1P2 cells and activation of endogenous S1P2 receptors in fibroblasts translated into profibrotic responses (Sobel et al., 2013). Since FTY720-P is often described as an S1P1,3,4,5 receptor agonist lacking activity at S1P2 receptors (Brinkmann et al., 2002, 2010), we further characterized the signaling pathways that are induced by stimulation of S1P2 receptors with FTY720-P in comparison with other S1P receptor agonists. We found that FTY720-P–induced S1P2 receptor signaling was detected by impedance-based assays in recombinant CHO-S1P2 cells and human NHLF-derived myofibroblasts, but not by classic second messenger assays, which monitored the increase or inhibition of cAMP accumulation or calcium release. To characterize the impedance response, we studied the effect of Rho/ROCK inhibition and Gα12/13 knockdown on FTY720-P–induced CHO-S1P2 signaling and demonstrated Gα12/13/Rho/ROCK dependency. Moreover, in CHO-S1P2 cells, FTY720-P, like the natural ligand S1P, directly activated Rho and induced a response in SRF-RE reporter gene assays, which are used to study Gα12/13–mediated activation of Rho/Rock signaling.

![Fig. 8](image-url). Analysis of NHLF-derived myofibroblast contraction induced by S1P receptor agonists using cell contraction assays. (A) Collagen gel lattices with NHLF-derived myofibroblasts were stimulated with FTY720-P (FTY-P), S1P, ponesimod (pones.), or LPA (all 5000 nM) after release of the gel lattices from the well, and pictures were taken after 30 minutes. (B) Collagen gel lattices with NHLF-derived myofibroblasts were stimulated with FTY720-P, S1P, ponesimod, or LPA (all 40–5000 nM) after release of the gel lattices from the well. At 30 minutes, the gel lattice area was divided by the initial area of each gel and contraction is expressed as relative gel area compared with the vehicle control (control = 1) at 30 minutes. (C) Collagen gel lattices were pretreated with the inhibitors JTE-013 (0.5 µM) or GSK269962 (GSK; 80 nM) for 2 hours, then gels were released from the well and stimulated for 30 minutes with FTY720-P (5000 nM), S1P (500 nM), ponesimod (5000 nM), LPA (5000 nM), or vehicle. At 30 minutes, the gel lattice area was determined and divided by the initial area of the lattice and compared with the samples treated with the corresponding inhibitor in the absence of an agonist. Data in (B and C) show mean ± S.E.M. of three independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001, one-way analysis of variance, Dunnett’s post-test (B and C). anta., antagonist.
ROCK signaling (Liu and Wu, 2004; Cheng et al., 2010). Since, in contrast to S1P, FTY720-P did not activate calcium signaling in CHO-S1P2 cells, we conclude that FTY720-P, at least in CHO-S1P2 cells, behaved as a biased agonist that selectively activated Ga12/13/Rho/ROCK signaling in the absence of the other second messenger release. As expected, the selective S1P1 receptor modulator ponsetimod was inactive in impedance-based and second messenger assays probing S1P2 receptor activation. We then extended our findings to primary cells and could show that in human myofibroblasts, FTY720-P–stimulated S1P2 receptors also activated the Ga12/13/Rho/ROCK pathway, and that such myofibroblasts displayed S1P2 receptor– and Rho/ROCK-dependent contractile responses similar to those obtained with S1P.

**Label-Free Analysis of GPCR Signaling Complements Classic Second Messenger Assays.** Second messenger assays are established methods to characterize GPCR modulators. Adenylate cyclase assays and assay monitoring changes in intracellular calcium are reliable, cheap, fast, and sensitive methods, which also lend themselves to high throughput screening. These assays capture all of the G-protein pathways, with the exception of Ga12/13–mediated signaling. Measurement of Ga12/13 pathway activation is less well established and mostly relies on downstream readouts, such as activation of the Rho/ROCK pathway or SRF-RE reporter gene transcription (Liu and Wu, 2004; Siehler, 2009; Cheng et al., 2010). Since Ga12/13/Rho/ROCK activation is coupled to cytoskeletal changes, label-free methods measuring cellular shape change, such as impedance-based assays, are uniquely sensitive to detect this pathway. Bearing this in mind, it is clear that the selective activation of the Ga12/13/Rho/ROCK pathway by S1P2 receptors stimulated with FTY720-P was evident using impedance-based assay methodology, whereas it had been overlooked using the classic repertoire of GPCR assays. Detailed analysis of the literature, however, revealed three independent reports describing S1P2 receptor activity of FTY720-P using β-arrestin recruitment assays or other desensitization-related readouts (Mandala et al., 2002; Graler and Goetzl, 2004; Wetter et al., 2009).

Interestingly, the selective activation of Ga12/13/Rho/ROCK signaling via a GPCR in the absence of other second messenger modulation, as we observe it in recombinant and primary cells for the S1P2 receptor and FTY720-P, to our knowledge not been reported so far. In most cases, ligands induced at least one additional pathway, such as calcium release (Rajagopal et al., 2010; Kozasa et al., 2011), as was also seen here for the natural ligand S1P at recombinant S1P2 receptors. Taken together, the use of the impedance-based assay technology in addition to classic GPCR assays allowed us to make a more complete description of compound activities at recombinant and endogenous S1P2 receptors.

**Rho Pathway Activation via S1P2 Receptors Causes Phenotypic Changes in Primary Cells.** Although the activation of the Ga12/13/Rho/ROCK pathway is more difficult to measure than second messenger–generating pathways, its physiologic and pathophysiologic importance is nevertheless high. Rho/ROCK signaling results in cellular responses, including modulation of gene transcription, rearrangement of the myosin–actin cytoskeleton, cell contraction, and fibrosis (Siehler, 2009; Kozasa et al., 2011; Knipe et al., 2015). S1P is a well established activator of the Rho/ROCK signaling pathway and is linked to multiple phenotypic changes in fibroblasts and vascular smooth muscle cells (Hu et al., 2006; Siehler, 2009; Xiang et al., 2013). S1P was shown to induce fibroblast-to-myofibroblast transformation and increase collagen expression via S1P2 receptor–mediated Rho signaling in lung and cardiac fibroblasts (Urata et al., 2005; Gellings Lowe et al., 2009). Moreover, Hashimoto et al. showed an S1P/S1P2 receptor/Rho-mediated augmentation of fibroblast chemotaxis and suggest an important Rho-dependent role of S1P in tissue repair and, if deregulated, in fibrosis (Hashimoto et al., 2008). In vascular smooth muscle cells, Rho signaling is activated by S1P2 receptors (Hu et al., 2006) and Rho activation is known to synergize with calcium-inducing agents (e.g., endothelin-1 and angiotensin II) to induce vasoconstriction, a phenomenon termed calcium sensitization (Lee et al., 2004). Furthermore, as shown in this study, S1P2 receptor/ Rho activation through FTY720-P or SLP leads to myofibroblast contraction. Fibroblast contraction is a phenomenon observed during wound healing and in many fibrotic disorders. In fact, myofibroblast contraction directly contributes to tissue stiffness and organ dysfunction and further promotes fibrosis by activating latent, extracellular matrix–bound TGF-β1 (Wipf et al., 2007; Hinz et al., 2012; Knipe et al., 2015). FTY720-P is the active metabolite of FTY720/Gilenya, which is an approved drug for the treatment of multiple sclerosis. Improving the selectivity of future S1P1 receptor modulators and to avoid S1P2 receptor activation appears to be an attractive strategy to improve the potential safety of this highly interesting compound class. Furthermore, label-free technologies, such as the impedance method, represent important complementary techniques in the characterization of GPCR modulators and may uncover previously undetectable activation of potentially safety-relevant signaling pathways.

**Authorship Contributions:**

- **Participated in research design:** Sobel, Gatfield, Studer.
- **Conducted experiments:** Sobel, Monnier, Menyhart, Bolinger.
- **Performed data analysis:** Sobel, Monnier, Gatfield, Bolinger.
- **Wrote or contributed to the writing of the manuscript:** Sobel, Naylor, Gatfield.

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