Supplementary Materials and Methods

Receptors and cell lines. $S1P_{1R}$ cDNA (Missouri S&T cDNA Resource Center) was transfected into HEK293T cells and GeneBLAzer® CRE-*bla* CHO-K1 cells (LifeSciences), selected with G418 and single cell clones were screened for cAMP production with 5 μM forskolin, in the presence or absence of 1 μM S1P. A clone that produced robust inhibition of cAMP with S1P was further characterized by flow cytometry with an antibody to $S1P_{1R}$ (R&D, Clone #218713). Tango™ EDG6/$S1P_{4R}$-*bla* U2OS cells were obtained from Life Sciences. $S1P_{2R}$ GeneBLAzer® CRE-*bla* CHO-K1 and $S1P_{3R}$/Gα16 GeneBLAzer® NFAT-*bla* CHOK1 cells were described elsewhere (18). HEK293T cells stably expressing $S1P_{1R}$-GFP are previously described (19). All cell lines were maintained under standard tissue culture conditions according to manufacturer's recommendations. Dialyzed or charcoal stripped FBS was used in all media containing FBS. Membrane preparations for $S1P_{1R}$ and $S1P_{5R}$ were from Multispan, and for $S1P_{2R}$ and $S1P_{3R}$, cells were homogenized in the presence of DNAase I (Life technologies) and Complete Protease Inhibitor-EDTA free tablets (Roche), centrifuged at 25,000*g* for 60 minutes at 4°C, and re-suspended at 2-5mg/ml in 20 mM HEPES, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 0.1% fatty acid free BSA, pH 7.4.

$S1P_{1R}$ internalization assay. HEK293T cells stably expressing $S1P_{1R}$-GFP seeded at $4x10^4$ cells/well in 96-well trays were incubated with a range of agonist concentrations, in the presence of 10 μM cycloheximide for 1 hour at 37°C, in triplicate. Cells were either analyzed immediately, or washed four times in agonist-free media and allowed to recover for 24 hours at 37°C in media containing 1 μM cycloheximide. Cell surface $S1P_{1R}$ expression was analyzed by flow cytometry with $S1P_{1R}$ antibody (R&D, Clone #218713) or isotype control, and Alexa647-conjugated anti-IgG_{2B} antibody. Stained cells were released from the surface by incubation with trypsin, which was determined not to affect the $S1P_{1R}$ receptor staining. Cells were analyzed with a BD FACSarray.

Cytokine analysis. Colon tissue extracts were generated by solubilizing 200 mg frozen tissue in 0.5M Tris-HCl, 0.1% Triton X-100, pH 7.4 with protease inhibitor complete cocktail (Roche). Samples wee clarified by centrifugation and lysates analyzed using Meso Scale Discoveries SECTOR® Imager 2400.

Pharmacokinetics of RPC1063. All pharmacokinetic studies were conducted in fed female Sprague-Dawley rats or C57BL/6 mice. A total of 63 animals were used for all pharmacodynamic and pharmacokinetic studies. RPC1063 was formulated in 5% DMSO, 5% Tween-20, 90% 0.1N HCl (mouse pharmacokinetic studies, and all efficacy studies) or in 0.5% carboxymethylcellulose (rat pharmacokinetics). Oral dosing (5 mL/kg) was by gavage. Blood was collected into BD Microtainer tubes (dipotassium EDTA). Plasma proteins were precipitated with acetonitrile (3:1 v/v), centrifuged and supernatant was analyzed by LC/MS/MS analysis. Brains were homogenized in acetonitrile at a 3:1 (v:w) ratio using a Biospec Beadbeater-16 with 1 mm glass bead. For the tissue analysis, a standard curve was prepared using homogenized brain samples from untreated animals. An eight point standard curve of RPC1063 spanning a range of 0.14 nM to 300 nM was included with each bio-analytical run. The lower limit of quantification of the method was typically 0.14 nM. The acceptance criteria for inclusion in the standard curve was +/- 15% for all standards, except for the lowest level of quantification (LLOQ) for which +/- 20% was allowed. Data were collected and analyzed using Analyst

software version 1.5.1. Blood concentration versus time data were analyzed using non-compartmental methods (WinNonlin Phoenix version 6.2) to calculate all pharmacokinetic parameters. Standard Error (SE) was calculated using:

$$S = \sqrt{(WRSS)/(DF)}$$

where WRSS is the weighted residual sums of squares, and DF is the number of observations minus the number of zero weights and the rank of the variance/covariance matrix.

Flow cytometry. Blood was collected either by cardiac puncture or retro-orbital bleeds into EDTA and stored at 4°C until the time of analysis. Lymphopenia was assessed either by veterinary hemoanalyzer or flow cytometry. For flow cytometry, blood (5-10 µl) was dispensed into polypropylene 96-well round bottom plates and incubated with either Mouse BD Fc Block™ (BD Pharmingen #553141) or mouse anti-rat CD32 (BD Pharmingen #550271) for 10 minutes prior to the addition of Alexa Fluor647-rat anti-mouse CD4 antibody (BD Pharmingen #557681), APC-Cy7-rat anti-mouse CD8a antibody (BD Pharmingen #557654), APC-rat anti-mouse F4/80 (eBioscience #17-4801), APC-rat anti mouse-Ly6G/Gr-l (eBioscience #17-5931), PE-rat anti-mouse CD45RA/B220 (BD #553090), PECy5-mouse anti-rat CD4 (BD Pharmingen #554839) or APC-mouse anti-rat CD8a (eBioscience #17-0084) for 1 hour on ice, followed by erythrocyte lysis with BD Pharm Lyse™ buffer. For CCR7 staining, erythrocytes were lysed first, followed by Fc blocking and PE-rat anti-mouse CD197/CCR7 (BD Pharmingen #560682) for 30min at 37°C. The appropriate isotype matched controls were used to establish specific staining profiles. Samples were washed in 1% FBS, 0.1% NaN_3 in PBS and samples were analyzed with a BD FACSarray.

Scoring system for disease models:

MOG-induced EAE model in C57Bl6 mice:

Mice were scored daily, starting 7 days after immunization (Day 7) and continuing until Day 28.

Score	Clinical observations
0.0	No obvious changes in motor function compared to non-immunized mice. When picked up by base of tail, the tail has tension and is erect. Hind legs are usually spread apart. When the mouse is walking, there is no gait or head tilting.
0.5	Tip of tail is limp. When picked up by base of tail, the tail has tension except for the tip. Muscle straining is felt in the tail, while the tail continues to move.
1.0	Limp tail. When picked up by base of tail, instead of being erect, the whole tail drapes over finger. Hind legs are usually spread apart. No signs of tail movement are observed.
1.5	Limp tail and hind leg inhibition. When picked up by base of tail, the whole tail drapes over finger. When the mouse is dropped on a wire rack, at least one hind leg falls through consistently. Walking is very slightly wobbly.

2.0	Limp tail and weakness of hind legs. When picked up by base of tail, the legs are not spread apart, but held closer together. When the mouse is observed walking, it has a clearly apparent wobbly walk. One foot may have toes dragging, but the other leg has no apparent inhibitions of movement. - OR - Mouse appears to be at score 0.0, but there are obvious signs of head tilting when the walk is observed. The balance is poor.
2.5	Limp tail and dragging of hind legs. Both hind legs have some movement, but both are dragging at the feet (mouse trips on hind feet). - OR - No movement in one leg/completely dragging one leg, but movement in the other leg. - OR - EAE severity appears mild when picked up (as score 0.0-1.5), but there is a strong head tilt that causes the mouse to occasionally fall over.
3.0	Limp tail and complete paralysis of hind legs (most common). - OR - Limp tail and almost complete paralysis of hind legs. One or both hind legs are able to paddle, but neither hind leg is able to move forward of the hind hip. - OR - Limp tail with paralysis of one front and one hind leg. - OR - ALL of: Severe head tilting, Walking only along the edges of the cage, Pushing against the cage wall, Spinning when picked up by base of tail.
3.5	Limp tail and complete paralysis of hind legs. In addition to: Mouse is moving around the cage, but when placed on its side, is unable to right itself. Hind legs are together on one side of body. - OR - Mouse is moving around the cage, but the hind quarters are flat like a pancake, giving the appearance of a hump in the front quarters of the mouse.
4.0	Limp tail, complete hind leg and partial front leg paralysis. Mouse is minimally moving around the cage but appears alert and feeding. Often euthanasia is recommended after the mouse scores 4.0 for 2 days. However, with daily s.c. fluids some mice can recover to 3.5 or 3.0. When the mouse is euthanized because of severe paralysis, a score of 5.0 is entered for that mouse for the rest of the experiment.
4.5	Complete hind and partial front leg paralysis, no movement around the cage. Mouse is not alert. Mouse has minimal movement in the front legs. The mouse barely responds to contact. Euthanasia is recommended. When the mouse is euthanized because of severe paralysis, a score of 5.0 is entered for that mouse for the rest of the experiment.

5.0	Mouse is spontaneously rolling in the cage (euthanasia is recommended). - OR - Mouse is found dead due to paralysis. - OR - Mouse is euthanized due to severe paralysis.

TNBS model of inflammatory bowel disease:

The severity of IBD was scored using the following scoring system: maximum score = 10

1. Adhesions:
   a) none = 0
   b) minimal = 1
   c) involving several bowel loops = 2
2. Strictures:
   a) none = 0
   b) mild = 2
   c) severe, proximal dilatation = 3
3. Ulcers:
   a) none = 0
   b) linear ulceration < 1 cm = 1
   c) two linear ulcers < 1 cm = 2
   d) more sites of ulceration or one large ulcer = 3
4. Wall thickness:
   a) less than 1 mm = 0
   b) 1-3 mm = 1
   c) 3 mm = 2

Naïve $CD4^{+}CD45Rb^{hi}$ T cell adoptive transfer model in SCID mice.

For each H&E stained section, submucosal edema will be quantified by measuring the distance from the muscularis mucosa to the internal border of the outer muscle layer in a non tangential area thought to most representative the severity of this change. Mucosal thickness will also be measured in a non-tangential area of the section that best represented the overall mucosal thickness. This parameter is indicative of gland elongation and mucosal hyperplasia. In order to incorporate this parameter into the summed score, a hyperplasia score will be derived from the measurement as follows:

0= <250 µm
1= 250-349 µm
2= 350-449 µm
3= 450-599 µm
4= 600-699 µm
5= ≥700 µm

The extent of inflammation (foamy macrophage, lymphocyte and PMN infiltrate) will be assigned severity scores according to the following criteria (semiquantitative evaluation):

Normal=0
Minimal=1 (generally focal affecting 1-10% of mucosa or if diffuse then minimal)
Mild=2 (generally focal affecting 11-25% of mucosa or if diffuse then mild)
Moderate=3 (26-50% of mucosa affected with areas of gland loss replaced by inflammatory cell infiltrate, milder in remaining areas of mucosa)
Marked=4 (51-75% of mucosa affected with areas of gland loss replaced by inflammatory cell infiltrate, milder in remaining areas of mucosa)
Severe=5 (76-100% of mucosa affected with areas of gland loss replaced by inflammatory cell infiltrate, milder in remaining areas of mucosa)

The parameters reflecting epithelial cell loss/damage were scored individually using a percent area involved scoring method:

None=0
1-10% of the mucosa affected=1
11-25% of the mucosa affected=2
26-50% of the mucosa affected=3
51-75% of the mucosa affected=4
76-100% of the mucosa affected=5

Parameters that are scored using percent involvement included:
Colon glandular epithelial loss - this includes crypt epithelial as well as remaining gland epithelial loss. Colon Erosion-this reflects loss of surface epithelium and generally is associated with mucosal hemorrhage (reflective of the bleeding seen clinically and at necropsy).

The 3 important scored parameters (inflammation, glandular epithelial loss, erosion) were ultimately be summed to arrive at a sum of histopathology score, which indicates the overall damage and would have a maximum score of 15.

Inflammatory cell infiltrates in the colonic mucosa will be evaluated for approximate percent of neutrophils in the total infiltrate using the criteria below. The approximate percent of total will then be multiplied by the 0-5 inflammation score in an attempt to semi-quantify relative PMN infiltration across sections and animals.

0 = approx 0%
10 = approx 10%
25 = approx 25%
50 = approx 50%
75 = 75% or greater

This value was then multiplied by the inflammation score in an attempt to semiquantify relative PMN infiltration across sections and animals.