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Novel sphingosine kinase-1 inhibitor, LCL351, reduces immune responses in murine DSS-induced colitis

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Abstract

Sphingosine-1-phosphate (S1P) is a biologically active sphingolipid metabolite which has been implicated in many diseases including cancer and inflammatory diseases. Recently, sphingosine kinase 1 (SK1), one of the isozymes which generates S1P, has been implicated in the development and progression of inflammatory bowel disease (IBD). Based on our previous work, we set out to determine the efficacy of a novel SK1 selective inhibitor, LCL351, in a murine model of IBD. LCL351 selectively inhibits SK1 both *in vitro* and in cells. LCL351, which accumulates in relevant tissues such as colon, did not have any adverse side effects *in vivo*. In mice challenged with dextran sodium sulfate (DSS), a murine model for IBD, LCL351 treatment protected from blood loss and splenomegaly. Additionally, LCL351 treatment reduced the expression of pro-inflammatory markers, and reduced neutrophil infiltration in colon tissue. Our results suggest inflammation associated with IBD can be targeted pharmacologically through the inhibition and degradation of SK1. Furthermore, our data also identifies desirable properties of SK1 inhibitors.

Graphical Abstract

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Keywords

Inflammation; Sphingosine 1-Phosphate; Sphingosine Kinase; Sphingolipids; Inflammatory Bowel Disease

Introduction

Sphingolipids were once thought to solely play a structural role, but in the last three decades it has been convincingly shown that these lipids have biological consequences. There are three well-studied bioactive sphingolipids which include: ceramide (Cer), sphingosine (Sph), and sphingosine 1-phosphate (S1P). Cer and Sph have been associated with cell death and growth arrest while S1P is associated with a pro-survival and pro-inflammatory phenotype [1]. The biologies associated with S1P are generated either by signaling through S1P receptors (S1PRs), a family of 5 G-protein coupled receptors, or through binding to intracellular targets, that include HDAC [2]. S1P itself has numerous roles in biological responses including neurogenesis and angiogenesis [3], lymphocyte egress [4], and inflammation [5].

Sphingosine kinases (SKs) generate S1P through the transfer of the γ -phosphate from adenosine triphosphate to the primary hydroxyl of Sph. To date, two isoforms have been cloned and characterized – SK1 and SK2. These enzymes sit at a critical junction in sphingolipid metabolism as they function to balance between the growth arrest-inducing lipids Cer and Sph and the pro-survival lipid S1P. SK1 is ubiquitously expressed in many tissue types [6]. Additionally, SK1 has been reported to be involved in a number of inflammatory diseases including rheumatoid arthritis [7], asthma [8], and inflammatory bowel disease (IBD) [9]. Several studies have shown that in response to the systemic inflammatory cytokine tumor necrosis factor- α (TNF- α) SK1 becomes activated [10–12]. Additionally, SK1 has been shown to induce cyclooxygenase-2 (COX-2) expression and increase production of prostaglandin E2 in response to TNF- α [10, 13]. Our group has shown that SK1 plays a critical role in a mouse model of IBD and that human patients with ulcerative colitis have increased SK1 protein expression [9]. Furthermore, we have shown that there are distinct roles for hematopoietic and non-hematopoietic derived SK1/S1P in IBD [14].

Over the years, there has been a large effort in drug discovery for compounds that can inhibit SK activity. Many of the early inhibitors of SK were analogs of Sph. Since the recent elucidation of the structure of SK1 (reviewed in [15]) there has been a dramatic increase in the number of small-molecule compounds which have been identified as potential inhibitors (inhibitors are reviewed in [15, 16]). Despite this large effort, there are currently no SK1 inhibitors approved by the U.S. Food and Drug Administration (FDA) as therapeutic options for any disease.

IBD comes in two major forms: ulcerative colitis (UC) and Crohn's disease (CD); these two inflammatory diseases are estimated to affect 1–1.3 million people in the United States alone [17]. While the exact cause of IBD remains elusive, there are many solid implications into it being an autoimmune disease. Since current therapies, mainly anti-TNF antibodies, used to treat IBD often result in loss of response in a significant number of patients [18] and many of these IBD patients will eventually require surgical intervention, we hypothesize that targeting SK1 might provide a previously untapped therapeutic avenue. Therefore, we set out to identify inhibitors of SK1 which could abrogate symptoms of IBD. Here we present a novel Sph analog, LCL351, which can selectively inhibit SK1 both *in vitro* and in cells. Additionally, LCL351 treatments *in vivo* could reduce the inflammatory response via reduction in TNF α , CXCL1 and CXCL2 expression levels and a reduction in infiltrating neutrophils.

Materials and Methods

Synthesis of SK inhibitors

LCL351 (L-*erythro*-2-N-(1'-carboxamidino)-sphingosine hydrochloride) and LCL146 (D-*erythro*-2-N-(1'-carboxamidino)-sphingosine hydrochloride) were synthesized by the Lipidomics Shared Resource Core Facility at the Medical University of South Carolina (MUSC) as previously described [19].

Lipid Analysis by ESI-MS/MS

Advanced analyses of sphingolipid species were performed by the Lipidomics Facility at MUSC on a TSQ 7000, triple-stage quadrupole mass spectrometer (Thermo Finnegan, Waltham, MA, USA) operating in a multiple reaction monitoring (MRM) positive ionization mode, as described previously [20]. For endogenous lipid measurements mouse colon tissue, distal colon sections were placed into extraction buffer and lysed using a bead beater for two cycles for 40 seconds at 6.5 power level.

¹⁷C-labeling of sphingolipids

Wild type (WT) and SK1^{-/-} mouse embryonic fibroblasts, A549 lung cancer, and HT29 colon cancer cells were pretreated with indicated doses of LCL146 or 351 for 2 hours. Fifteen minutes prior to harvest, ¹⁷C-sphingosine (¹⁷C-Sph) was added to the media (1 μ M final concentration). Cells were harvested by scraping into ice-cold PBS, pelleted, and re-suspended in cell extraction solution (ethyl acetate/isopropanol/water, 60:30:10, v/v/v). Extraction and analysis were performed as described previously [21].

Fluorescent Sphingosine Kinase Assay

Omega NBD-labeled sphingosine (Avanti Polar Lipids, Alabaster, AL) was complexed with 4mg/mL bovine serum albumin (Sigma-Aldrich, St. Louis, MO). SK assays were performed as previously described [22] with slight modification. Briefly, recombinant human protein (Cayman Chemical Co., Ann Arbor, MI) was incubated with 10 μ M NBD-Sph, 1mM ATP, and 1X reaction buffer (5mM HEPES pH 7.4, 15mM MgCl₂, 0.05% Triton X-100, 10mM KCl) in a 100 μ L final reaction volume. After 1 hour of incubation, 100 μ L of a 1M potassium phosphate dibasic pH 8.5 solution was added followed immediately by quenching with

500 μ L of a chloroform:methanol (2:1) solution. Phases were separated by centrifugation for 5 minutes at 3000 RPM. The aqueous phase was removed and read in the Synergy HT plate reader (BioTek Instruments Inc., VT, USA) using a 96-well plate.

Analysis of Cell Cycle by Flow Cytometry

Cells were pretreated with LCL351, washed in cold PBS, scraped, and pelleted. Cell pellets were resuspended in -20°C 70% ethanol and placed at 4°C overnight. For analysis, cells were pelleted, ethanol-aspirated, and then resuspended in 0.5ml of hypotonic staining solution (0.25g of sodium citrate, 0.75ml of Triton X-100, 0.025g of propidium iodide, 0.005g of ribonuclease A in 250 ml of water) for 30 min. Cell cycle analyses were performed in the MUSC Flow Cytometry Facility.

Chemicals and reagents for analysis of LCL351

All solvents and water were HPLC grade and were purchased from Fisher Scientific (USA). The internal standard (IS), 17C-Sph, was purchased from Avanti Polar Lipids (Alabaster, AL) and LCL351 was synthesized in-house Lipidomics Shared Resource Core Facility at MUSC (Charleston, SC). Stock solutions were prepared in 1mM ammonium formate in methanol containing 0.2% formic acid. All experiments were performed at the Pharmacokinetics and Drug Metabolism Core of the MUSC (Charleston, SC).

Method for determination of LCL351

A standard curve ranging from 0.05 – 0.8 $\mu\text{g/ml}$ for LCL351 was generated in 1mM ammonium formate in methanol containing 0.2% formic acid with $R^2=0.996$. The IS (final concentration 0.5 $\mu\text{g/ml}$) was added to the standards and serum samples, after which samples were extracted with iso-propanol:ethyl acetate (15:85; v:v). Following centrifugation, the organic phase was transferred to a new glass vial and the aqueous phase was acidified using 100 μl of formic acid. The samples were centrifuged again and the upper organic phases combined. After drying under nitrogen, the extracts were reconstituted in 1 mM ammonium formate in methanol containing 0.2% formic acid and transferred to borosilicate HPLC vials (MicroSolv, Eatontown, NJ) with maximum recovery inserts (Waters, Milford, MA) and 7.5 μl was injected for UPLC-MS/MS analysis. The lower limit of detection and recovery were 0.002 $\mu\text{g/ml}$ and 110% respectively.

UPLC-MS/MS analysis of LCL351 from in vivo samples

An Acquity UPLC coupled to a Quattro Premier XE mass spectrometer (Waters, Milford, MA) was used to measure LCL351 concentrations. Chromatographic separation was performed on an Acquity UPLC HSS C18 $2.1 \times 100\text{mm}$ (1.8 μm) column preceded by an Acquity UPLC HSS C18 (1.8 μm) pre-column. Samples were eluted over 6.5 min and mobile phase A consisted of 2mM ammonium formate in water containing 0.2% formic acid with a flow rate of 0.4ml/min. Mobile phase B consisted of 1mM ammonium formate in methanol containing 0.2% formic acid. The mass spectrometer was operated in positive ion mode with capillary voltage 3.1kV, source temperature 120°C , desolvation temperature 300°C and nitrogen gas flow at 700L/Hr. Data acquisition was performed using MassLynx 4.1 and quantification using QuanLynx 4.1 (Waters, Milford, MA). The multiple reaction monitoring

(MRM) transitions were as follow: IS m/z 286.47 → 268.3 and LCL351 m/z 342.47 → 324.3. The cone voltages were 25V and 45V, and the collision energy 12V and 20V respectively.

Pharmacokinetic/Pharmacodynamic Studies

C57BL/6 wild type (WT) male mice were purchased from Jackson Laboratories (Bar Harbor, ME) at 8 weeks of age. Animals were maintained under standard laboratory conditions with ad libitum access to food and water. All animal procedures were approved by the MUSC Institutional Animal Care and Use Committee and followed the guidelines of the American Veterinary Medical Association. Mice were intraperitoneally (i.p.) injected with 6mg/kg LCL351 for indicated times. The concentration was determined based on efficacy in cells which was then translated into an in vivo concentration. Plasma and tissues were collected and analyzed for LCL351 and sphingolipid levels as described above.

LCL351 and Induction of colitis

On days 0, 1, 2, 3, and 4 mice were i.p. injected with 6mg/kg LCL351 or vehicle (10% ethanol) and acute colitis was induced by adding 5% (w/v) DSS (MP Biomedicals, Inc., Solon, OH, USA) to the drinking water for all 5 days. DSS solutions were monitored to ensure equal consumption among treatment groups. Untreated mice were given regular drinking water. Parameters of disease, including splenomegaly, colon length and weight loss were determined as previously described [23].

Real-time RT-PCR and Analysis

RNA was extracted from colon tissues using the Qiagen RNeasy kit per the manufacturer's instructions. RNA was reverse transcribed into cDNA using 0.5µg RNA, Oligo_{DT} primers and SuperScriptIII from Invitrogen (Carlsbad, CA, USA). Real-time RT-PCR was performed on an ABI 7500 to quantify mRNA levels of COX-2. The standard real-time RT-PCR reaction volume was 20µl, including 10µl iTaq Universal Probe master mix PCR reagents (Biorad, Hercules, CA, USA), 4µl cDNA template, 1µl TaqMan Gene Expression Assay primers, and 5µl water. The RT-PCR steps were as follows: 2 minutes at 95°C, followed by cycles (n=40) consisting of 15s melt at 95°C, 60s annealing/extension at 60°C and a final step of 1 min incubation at 60°C. All reactions were performed in triplicate. The data were analyzed using Q-Gene software [24] and expressed as mean normalized expression (MNE). MNE is directly proportional to the amount of RNA of the target gene relative to the amount of RNA of the reference gene β-actin.

Histology and immunohistochemistry assays

Colons were flushed with cold PBS and fixed in 10% neutral-buffered formalin (Sigma) at 4°C. Paraffin embedded sections (5µm) were deparaffinized immunohistochemistry was performed on sections heated for 10 minutes in 10mM citrate buffer (pH 6.2) for antigen retrieval. After endogenous peroxidase removal (3% H₂O₂ 5 min), sections were incubated with anti-neutrophil antibodies raised against rat (Abcam NIMP-R14 [ab2557], 1/200 dilution). Secondary biotinylated anti-rat antibodies (Vector Laboratories Inc) (1/200

dilution) were detected using the Vectastain ABC kit (Vector Laboratories Inc). Counter staining was performed with hematoxylin.

Statistical analysis

Statistical analyses were performed using two-way ANOVA with Bonferroni post-hoc tests or students t-test for LCL351 and DSS effects. p -values <0.05 were considered significant.

Results

Identification of a novel sphingosine kinase inhibitor

In an effort to discover novel and selective SK1 inhibitors, a number of structurally diverse Sph analogs were generated [19]. The most successful analogs resulted from modifications of the polar head group of Sph, which were based on an “amine-guanidine switch” [25], and the changes of its stereochemistry resulting in LCL146 and LCL351 (Figure 1A). These two guanidine analogs of Sph represent the enantiomeric pair of the erythro diastereoisomers, differing by the stereochemistry at C2 and C3 positions of the sphingolipid backbone. Both compounds inhibited 17C-S1P generation in A549 cells with LCL351 being the more potent of the two analogs (Figure 1B). Lipid levels in cells treated with LCL351 were compared to those treated with the small molecule inhibitor SKi-II. LCL351, similar to SKi-II, reduced 17C-S1P in wild-type (WT) mouse embryonic fibroblasts (MEFs) (Figure 1C). Interestingly, unlike SKi-II, LCL351 significantly increased both 17C-Sph and 17C-ceramide levels in WT MEFs (Figures 1D and 1E). Since LCL351 was the more potent of the two guanidine compounds, this was the compound that was used for all subsequent experiments.

LCL351 is a selective SK1 inhibitor *in vitro* and in cells

To determine whether LCL351 was inhibiting SK1, SK2, or both isoforms *in vitro* and cellular SK activity assays were performed. Recombinant SK1 and SK2 were used in fluorescence-based *in vitro* assays. We examined the ability of LCL351 to inhibit either isoform of SK and determined LCL351 has a half-maximal inhibitory concentration for SK1 (LogIC_{50}) of -5.258 ± 0.08817 (approximately $5.5\mu\text{M}$) whereas the LogIC_{50} for SK2 was -4.244 ± 0.1124 (approximately $57\mu\text{M}$) (Figures 2A and 2B). We used the IC_{50} values to estimate the K_i values (Figure 2B) based on the Cheng-Prusoff equation using a web-based software [26] which takes into account the concentrations of enzyme and substrate, the substrate K_m , and the IC_{50} . Using this tool, we estimated the K_i values for SK1 and SK2 to be $4.36921\mu\text{M}$ and $46.42815\mu\text{M}$ respectively (Figure 2B). Both the IC_{50} and the estimated K_i values demonstrated that the selectivity of LCL351 for SK1 over SK2 was greater than 10-fold. Moreover, 17C-Sph incorporation into 17C-S1P was evaluated to further define LCL351 as an SK1 selective inhibitor in cells. MEFs isolated from WT, SK1^{-/-}, or SK2^{-/-} mice were pretreated with LCL351 for 2 hours and then labeled with $1\mu\text{M}$ 17C-Sph. WT MEFs demonstrated a decrease in 17C-S1P production as well as an increase in 17C-Sph in response to LCL351 in a dose dependent manner (Figures 2C and 2D). In the SK1^{-/-} MEFs, where only SK2 is present, there was no effect on 17C-S1P or 17C-Sph. Moreover, in the SK2^{-/-} MEFs, where only SK1 is present, there was both a significant decrease of 17C-S1P and a significant increase in 17C-Sph (Figures 2C and 2D).

Several SK1 inhibitors have been reported to influence the protein level of SK1 and cell viability; therefore, we assessed the effects of LCL351 on viability and SK1 levels in cells. CaCo-2 cells (a colon cancer cell line chosen because SK1 has been shown to play a pivotal role in colitis and colitis-associated cancer) were treated with either LCL351 or SKi-II followed by SK1 protein level assessment via immunoblot. Both LCL351 and SKi-II decreased SK1 at the protein level although LCL351 was slightly less efficient than SKi-II at 10 μ M (Figure S1A). Cell viability was also assessed; LCL351 did not affect cell viability until 100 μ M, approximately 20-fold higher than the IC₅₀ (Figure S1B). Furthermore, upon analysis of cell cycle, LCL351 did not alter G1 and G2/M populations but did induce a slight and significant decrease in the S-phase population (Figure S1C).

Systemic effects of LCL351 treatment on DSS-induced colitis in vivo

To begin determining the efficacy *in vivo*, we examined the plasma half-life. Using 6mg/kg in mice, the plasma half-life of LCL351 was relatively short at approximately 30–45 minutes (Figure S2A); however, this was still able to significantly decrease plasma S1P levels (Figure S2B) at the early time points after which they returned to normal levels. There was an increase in Sph which was not significant and Cer levels remained constant (Figures S2C and S2D). Moreover, the residence time of LCL351 in tissues was much longer as compared to the plasma half-life, suggesting that LCL351 can be easily cleared by the liver but stays significantly longer within tissues such as the colon (Figure 3A).

To determine the effect of LCL351 on acute DSS-induced colitis, mice were intraperitoneally injected with 6mg/kg LCL351 once per day for days 0–4 in conjunction with 5% DSS in the drinking water to induce colitis followed by euthanasia on Day 5 (Figure 3B). Physical parameters of DSS-induced colitis including weight loss, colon shortening, and splenomegaly were measured in these mice. In vehicle treated mice, DSS administration resulted in significant weight loss at Day 5 of DSS treatment. Notably, in LCL351 treated mice DSS did not induce a significant amount of weight loss at Day 5 (Figure 3C). Splenomegaly is commonly associated with colitis and was evident in vehicle treated mice after DSS administration (Figure 3D). However, this DSS-induced increase in spleen size was abrogated with LCL351 treatment (Figure 3D). Mice administered regular drinking water did not demonstrate weight loss or splenomegaly after 5 days of treatment with either LCL351 or vehicle. Another physical parameter of colitis is colon shortening, which was decreased upon DSS administration in both vehicle and LCL351 treated mice (Figure 3E). Histologic damage was assessed based on inflammation, crypt damage, and percent of the colon involved. LCL351 slightly, but not significantly, decreased overall colonic damage in response to DSS (data not shown). Together these data suggest that LCL351 administration partially protects against DSS-induced colitis.

Blood loss from the colon into the stool is common in colitis as the structure of the colon is compromised in inflamed areas. Since the Hemocult® test is not quantitative, several other parameters were measured which could quantitate blood loss. Using complete blood counts (CBCs), red blood cells (RBCs), hematocrit, hemoglobin, and platelet numbers were assessed. In the vehicle treated mice there was a significant reduction in the number of RBCs in the blood upon DSS administration (Figure 4A). However, mice treated with

LCL351 were protected from this loss of RBCs. In accordance with the RBCs, hemoglobin and hematocrit were both decreased in vehicle treated mice upon DSS administration, and this was rescued by LCL351 (Figure 4B and 4C).

Sphingolipids in the blood were assessed to determine the effects of LCL351 on circulating levels. S1P levels were increased upon DSS administration in vehicle treated mice; however, while S1P levels in LCL351 treated mice were not increased upon DSS administration. S1P levels were basally slightly higher in untreated mice administered LCL351 (Table S1). Overall, LCL351 protected from blood loss, reduced weight loss and splenomegaly in DSS-induced colitis.

Effects of LCL351 in colon tissue in DSS-induced colitis in vivo

We have previously shown that mice deficient in SK1 were protected from increased neutrophil recruitment to colon tissues upon DSS-induced colitis. Therefore, we set out to determine if tissue inflammatory responses or neutrophil recruitment to the colon would be altered or prevented by LCL351 treatment. In colon tissue, expression of chemokines C-X-C motif ligand (CXCL) 1 and CXCL2 were determined as these chemokines are involved in the early recruitment of neutrophils [27]. CXCL1 and CXCL2 mRNA expression was decreased by LCL351 treatment in response to DSS (Figures 5A and 5B). Additionally, TNF α mRNA was significantly increased in response to DSS and was somewhat abrogated by LCL351; however, the decrease was not significant (Figure 5C). S1P levels in colon tissue were decreased in mice treated with DSS and LCL351 (Figure 5D), suggesting that, in response to DSS, S1P generation was impaired. Additionally, treatment with LCL351 decreased endogenous C16-Cer as well as total Cer levels basally in colon tissues. Yet upon DSS treatment C16-Cer and total Cer levels were elevated in mice that received LCL351 (Table S3). These data suggest that LCL351 alters sphingolipid levels basally and prevents S1P generation in the setting of DSS-induced colitis.

In addition, immunohistochemical staining was used to assess neutrophil infiltration into colon tissue. We show representative images of colon tissue from n = 4. Mice treated with vehicle exhibited significant increases in neutrophils infiltrating into inflamed colon tissue after DSS administration (Figures 5E and 5F).

Treatment with LCL351 abolished this increase in neutrophils into inflamed tissues upon DSS (Figures 5G and 5H). In sections of colon with the most normal crypt structure, the number of neutrophils was similar between LCL351 and vehicle treatments (Figures 5E and 5G). However, examination of sections where crypt structure has been lost, indicating inflamed areas, revealed fewer neutrophils in the LCL351 treated mice compared to the vehicle treated mice (Figures 5F and 5H). We believe that this indicates that inhibition of SK1 in the tissue impairs the ability of WBCs, specifically neutrophils, to infiltrate into the tissue. Interestingly, CBCs showed that mice treated with DSS and LCL351 had an increased number of circulating white blood cells (WBCs) (Table S2). Altogether, these data suggest that while WBCs may be increased in circulation, these cells may not be properly recruited to sites of inflammation in tissues.

Discussion

In this study, we present proof-of-concept for pharmacologically targeting SK1 in IBD, characterizing this novel inhibitor and expanding our previous studies using genetic approaches. We show a novel SK1 inhibitor, LCL351, which inhibited SK1 activity *in vitro* ($IC_{50} \sim 5.5 \mu M$) with a 10-fold selectivity for SK1 over SK2. Additionally, this novel SK1 inhibitor reduced immune responses *in vivo* in a well-established model of colitis. In cells, we demonstrated that LCL351 selectively inhibited SK1 with no inhibition of SK2 at the concentrations used in this study. There were no adverse side effects of this inhibitor on cell death or cell cycle despite LCL351-induced degradation of SK1 at the protein level, which is important as induction of cell death might exacerbate inflammatory responses. It is of note that our C17-Sph treatment of cells does not give a complete overview in possible changes in sphingolipids. LCL351 *in vivo* reduced plasma S1P levels in mice even with its admittedly short half-life. However, LCL351 does have a longer residence time in tissues and can decrease tissue S1P levels, which could be beneficial for its role in protecting from tissue inflammation. In mice with DSS-induced colitis LCL351 protected from weight loss and splenomegaly, as well as blood loss as indicated by RBC counts, hematocrit, and hemoglobin. Even though LCL351 treatment only slightly attenuated induction of $TNF\alpha$ in colon tissue, neutrophil infiltration was ablated by this SK1 selective inhibitor. These data demonstrate effects of a novel inhibitor of SK1 which degrades the protein but does not induce cell death, an ideal situation for the treatment of inflammatory diseases.

Recently, several SK inhibitors have been identified to target SKs in many different diseases including inflammatory diseases and cancers. However, there are currently no direct SK inhibitors with FDA approval for the treatment of any disease. Some of the identified inhibitors inhibit both SK1 and SK2 whereas others are selective for one isozyme over the other. Many of these inhibitors (reviewed in [16, 28]) target the Sph binding site of SKs, with one report of an ATP-competitive SK inhibitor [29]; however, targeting the ATP-binding site might prove difficult for specificity as it has for many protein kinase inhibitors, but this remains to be determined.

Many SK inhibitors (including LCL351) have dual mechanisms of action: 1) Sph-competitive inhibition of kinase activity and 2) proteolysis of SK1. These dual effects of these inhibitors make it difficult to determine whether the effect is due to loss of activity or to the loss of the protein itself. This is a critical issue for those who study SK1 which needs to be resolved in order to determine which of these mechanisms is important for effects seen with the treatment of SK1 inhibitors. Pharmacological inhibition of SK1 with several different inhibitors has been shown to have no effect on the viability of several different cancer cell lines [30, 31]. Whereas knockdown of *Sphk1* with siRNA shows that SK1 may be important in cell survival and the promotion of cell growth [32, 33]. Thus, interpreting data using pharmacological inhibitors or silencing technologies must be done very carefully as there may be both kinase-mediated and non-kinase mediated biologies of SK1. Understanding this paradigm will be extremely important in studies geared at generating novel therapeutics and inhibitors for the treatment of disease.

Despite the work still needed to be done to understand the aforementioned SK1 paradigm, the field is pushing forward to find inhibitors of SK1 and SK2 for the treatment of diseases. There have been several studies looking at IBD treatment via inhibition of SKs [34, 35]. The main inhibitor used in these studies, ABC294640, was subsequently found to inhibit SK2 [36]. More recently, another group has published on inhibition of SKs in IBD [37]; however, the inhibitor was equally as effective, if not more effective, against SK2 as it was for SK1. Our lab has used genetic techniques to understand and implicate the role of SK1 specifically in IBD [14, 38].

It is interesting to examine the similarities between genetic ablation of SK1 in the context of DSS [14, 38] and the pharmacological inhibition of SK1 (this study). DSS-induced splenomegaly was reduced with both LCL351 treatment and in SK1^{-/-} mice transplanted with WT bone marrow [14]. Colon shortening occurs in SK1^{-/-} mice and LCL351 mice in response to DSS [38]. However, both LCL351 and SK1^{-/-} mice were partially protected from weight loss. Additionally, treatment with LCL351 mimicked SK1^{-/-} mice by rescuing blood loss associated with DSS-induced colitis. LCL351 treatment, like the SK1^{-/-} mice, slightly mitigated the increase in expression levels of the systemic inflammatory marker TNF α [14]. Moreover, like the SK1^{-/-} mice, LCL351 treatment resulted in less infiltrating granulocytes (specifically neutrophils) into sites of inflammation. Together with our previous studies, the data presented here clearly indicate that SK1 is a valid therapeutic target for IBD.

The efficacy of LCL351 in the reduction of inflammation in DSS-induced colitis, can perhaps be explained through its pharmacokinetic and pharmacodynamic properties. LCL351 is cleared very quickly from the plasma of mice but has a significantly longer residence time in tissues, such as colon. This creates a unique situation where levels of S1P are not affected in circulation over long periods of time. At the same time, LCL351 exhibits increased residence time in colon tissues and may exert its effects on SK1 protein present in the tissue. S1P has been shown to be important for egress of many different hematopoietic cells from tissues into the blood [39, 40]. LCL351 elevated circulating S1P levels basally; however, this was not significant and LCL351 prevented DSS-induced increases in S1P (Table S1). While we know that the S1P gradient is important for egress of WBCs from secondary lymph tissues and other vascular and immunological biologies (reviewed in [41]), we know relatively little about the role of S1P in WBC infiltration into inflamed tissues [42]. However, we hypothesize that the number of circulating WBCs are increased in response to LCL351, partly due to the inability of LCL351 to inhibit SK1 in circulation (half-life 30–45 minutes; slight basal increase in S1P). Furthermore, LCL351 dependent decreases in S1P in tissue in response to DSS could be increasing the gradient between circulating S1P and inflamed tissues. The reduction in the neutrophil chemoattractants CXCL1 and CXCL2 compound the issue of the altered S1P gradient. In effect, this increased gradient and decreased recruitment signals could act to trap WBCs in the circulation and prevent their recruitment into sites of tissue inflammation (Figure 6).

The increasing S1P in circulation yet decreasing tissue levels of S1P in inflammation, could also explain the prevention of blood loss during DSS-induced colitis by LCL351. Erythrocytes are a major source of S1P in the blood, although how they are regulated

remains somewhat unclear [43]. However, there is an abundance of evidence that shows S1P is critical for maintaining endothelial barrier function (reviewed in [44]). Blood loss, as quantified by RBC counts, hematocrit, and hemoglobin, was rescued by LCL351 in response to DSS. Since the plasma half-life of LCL351 is short, S1P concentrations remain significant in the blood, potentially resulting in enhanced endothelial barrier function and significant reduction of blood loss.

Conclusions

Overall, we demonstrate in this study that the novel and selective inhibitor, LCL351, selectively inhibited SK1 without affecting SK2 activity. Additionally, in proof-of-concept experiments, we have shown that pharmacological modulation of SK1 can alleviate several manifestations of IBD and mimic certain aspects of the SK1^{-/-} mice in response to DSS. These data suggest that inhibition of SK1 activity may be sufficient to protect from certain aspects of inflammation without resulting in detrimental cytotoxic effects. This work indicates that there are favorable properties which SK1 inhibitors should possess for inflammatory disease such as, short plasma half-life but very high volume of distribution so it can remain in the tissue rather than be cleared from the plasma by the kidneys and liver. Future studies with this novel SK1 inhibitor will be focused on improving the potency and pharmacokinetic and pharmacodynamics properties, potentially improving the protective effects in IBD.

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Abbreviations

SK	Sphingosine Kinase
Sph	Sphingosine
S1P	Sphingosine 1-phosphate
Cer	Ceramide
17C-Sph	17 carbon chain sphingosine
17C-S1P	17 carbon chain sphingosine 1-phosphate
DSS	dextran sodium sulfate

IBD	inflammatory bowel disease
MEFs	mouse embryonic fibroblasts
TNF-α	tumor necrosis factor alpha
CXCL1/2	Chemokine C-X-C Motif Ligand 1/2

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Highlights

- Novel sphingosine analog, LCL351, inhibits SK1 in cells (10X more selective for SK1 over SK2 *in vitro*).
- LCL351 treatment *in vivo* demonstrated protection from weight loss, blood loss, and splenomegaly in a mouse model of IBD.
- LCL351 reduced induction of inflammatory cytokines and neutrophil infiltration in colon tissue in a mouse model of IBD.

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Figure 1. The novel SK inhibitor LCL351 prevents C17-S1P generation in cells

A) Structures for SK1 inhibitors LCL146 and 351. **B)** A549 cells were treated with either vehicle (10% ethanol, VEH) or LCL compounds for 2 hours, labeled with 1 μ M C17-sphingosine 15 minutes prior to harvest, and C17-sphingolipids measured by LC-MS/MS. **C–E)** MEFs were treated with either vehicle, LCL351, or SKi-II for 2 hours, labeled with 1 μ M C17-sphingosine 15 minutes prior to harvest, and C17-sphingolipids were measured by LC-MS/MS. Data represent mean fold change from vehicle $\pm$ SEM for n = 3; *p<0.05, **p<0.01 as compared to VEH.

Sphingosine Kinase Isoform	Calculated LogIC ₅₀ s	95% Confidence Intervals for LogIC ₅₀ s	Estimated K _i (μM)
Sphingosine Kinase 1	-5.258 ± 0.08817	-5.439 to -5.077	4.36921
Sphingosine Kinase 2	-4.244 ± 0.1124	-4.475 to -4.014	46.42815

Figure 2. LCL351 selectively inhibits SK1

A) Recombinant human proteins, SK1 and SK2 were treated with LCL351 and tested for inhibition; IC₅₀ concentrations of LCL351 for SK1 and SK2 were determined. Data represent n=3 ± S.E.M. B) calculated IC₅₀s from A) along with the 95% confidence intervals and estimated K_i values. C) and D) WT, SK1^{-/-} or SK2^{-/-} cells were treated with indicated doses of LCL351 or VEH for 2 hours, labeled with 1 μM C17 Sph, and lipids measured by LC/MS/MS. Data represent mean fold change from vehicle ± SEM for n = 3; *p<0.05, **p<0.01 as compared to VEH.

Figure 3. Efficacy of LCL351 in acute DSS-induced colitis

A) Tissue content of LCL351 in different organs post-injection. Data represent the mean of $n=6 \pm \text{SEM}$. B) Scheme for the administration of both DSS and LCL351 in C57BL6 mice. 6mg/kg LCL351 was injected i.p. daily during 5% DSS administration in drinking water. C) Body weight, D) spleen weight, and E) colon length were measured. Data represent mean $\pm$ SEM, $n=6$; * $p<0.05$, *** $p<0.001$, **** $p<0.0001$ as compared to the respective untreated control. # $p<0.05$ as compared to VEH/DSS.

Figure 4. LCL351 rescues blood loss associated with DSS induced colitis

Blood was collected from mice at Day 5 and was analyzed for A) red blood cell (RBCs) counts, B) hemoglobin, and C) hematocrit. Data represent the mean ± SEM; n = 7. ****p < 0.0001 as compared to the respective untreated control.

Figure 5. LCL351 reduces colon S1P and recruitment of neutrophils in response to DSS
 Mice were injected with 6mg/kg LCL351 or VEH daily and administered 5% DSS in drinking water for 5 days and A) CXCL1, B) CXCL2, and C) TNFα mRNA levels were measured in colon tissue using real-time RT-PCR and normalized to β-actin. D) S1P levels from colon tissue were determined using LC/MS/MS and normalized to total lipid phosphate. Data represent mean ± SEM; n = 3; *p<0.05, ***p<0.001 as compared to the respective untreated control. E–H) Representative images of immunohistochemical staining for neutrophils n = 4. E) and G) represent tissues where DSS-induced minimal tissue damage.

F) and H) represent tissues with significant DSS-induced structural damage. Scale bars are 50 μm , with 20x and 40x magnification, respectively.

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Figure 6. Proposed model for inhibition of SK1 by LCL351

LCL351 exhibited a short plasma half-life, reducing S1P in circulation only transiently. This potentially allowed vascular integrity to remain intact and resulted in less blood loss in LCL351 treated mice upon DSS administration. Residence time of LCL351 was significantly longer in the colon tissues, resulting in decreased S1P generation and neutrophil recruitment into colon tissues. Together these data suggest that tissue inhibition of SK1 may present a valid therapeutic target in IBD.