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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.

1. 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

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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[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* reduced the inflammatory response via reduction in TNFα, CXCL1 and CXCL2 expression levels and in infiltrating neutrophils.

2. Materials and Methods.

2.1. 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].

2.2. 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.

2.3. 17-Carbon 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 μm 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].

2.4. Fluorescent Sphingosine Kinase Assay:

Omega NBD-labeled sphingosine (Avanti Polar Lipids, Alabaster, AL) was complexed with 4 mg/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 μM NBD-Sph, 1 mM ATP, and 1X reaction buffer (5 mM HEPES pH 7.4, 15 mM $MgCl_2$, 0.05% Triton X-100, 10 mM KCl) in a 100 μL final reaction volume. After 1 hour of incubation, 100 μL of a 1 M potassium phosphate dibasic pH 8.5 solution was added followed immediately by quenching with 500 μL 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.

2.5. 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 0.5 ml of hypotonic staining solution (0.25 g of sodium citrate, 0.75 ml of Triton X-100, 0.025 g of propidium iodide, 0.005 g of ribonuclease A in 250 ml of water) for 30 min. Cell cycle analyses were performed in the MUSC Flow Cytometry Facility.

2.6. 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 1 mM 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).

2.7. Method for determination of LCL351

A standard curve ranging from 0.05–0.8 μg/ml for LCL351 was generated in 1 mM ammonium formate in methanol containing 0.2% formic acid with $R^2 = 0.996$. The IS (final concentration 0.5ug/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 0.002ug/ml and 110% respectively.

2.8. 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 × 100 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 2 mM ammonium formate in water containing 0.2% formic acid with a flow rate of 0.4 ml/min. Mobile phase B consisted of 1 mM ammonium formate in methanol containing 0.2% formic acid. The mass spectrometer was operated in positive ion mode with capillary voltage 3.1 kV, 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 25 V and 45 V, and the collision energy 12 V and 20 V respectively.