Online Data Supplement

Increasing Sphingolipid Synthesis Alleviates Airway Hyperreactivity

Andrea F.Heras, Arul Veerappan, Randi B. Silver, Charles W. Emala, Tilla S. Worgall, Jose Perez-Zoghbi, Stefan Worgall

Supplementary Material & Methods

Quantitative Sphingolipid Determination

Sphingolipids were quantified in cells and homogenates of murine lungs and precision cut lung slices by high pressure liquid chromatography electrospray ionization tandem mass spectrometry (HPLC-MS/MS) using minor modification of a described method(E1). The method is validated for 5 dihydroceramides: (d18:0/16:0 d18:0/18:0, d18:0/18:1, d18:0/24:0, d18:0/24:1), 6 ceramides (d18:1/C16:0, d18:1/C18:0, d18:1/C20:0, d18:1/C22:0, d18:1/C24:0, d18:1/C24:1), 4 sphingomyelins (SM d18:1/C16:0, SM d18:1/C18:0, SM d18:1/C18:1, SM d18:1/C24:1), and 4 long-chain bases: sphingosine (SO d18:1), sphinganine (SA d18:0), sphingosine-1-phosphate (S1P d18:1), sphinganine-1-phosphate (Sa-1-P d18:0). 25 ul serum or whole blood were extracted by vortexing overnight in 900 ul dichloromethane / methanol (1:1) with addition of internal standard (N-lauroyl-D-erythro-sphingosylphosphorylcholine). After centrifugation to precipitate cell debris, an aliquot was transferred into an Agilent 1200 HPLC (Agilent Poroshell 120 column) linked to an Agilent 6430 triple quadrupole mass spectrometer. Mobile phase A consisted of methanol/water/chloroform/formic acid (55:40:5:0.4 v/v); Mobile phase B consisted of methanol/acetonitrile/chloroform/formic acid (48:48:4:0.4 v/v). After pre-equilibration for 6 sec, the gradient was increased gradually to 60% mobile phase B and 100% mobile phase B that was held for 1.9 min. With a flow rate is 0.6 mL/min, the duration of the entire run was 9.65 min. We used the Mass Hunter optimizer and pure synthetic standards

(Avanti Polar Lipids) to determine optimum fragmentation voltage, precursor/ product ions and m/z values. Peak calls and abundance calculations were obtained with MassHunter Workstation Software Version B.06.00 SP01/Build 6.0.388.1 (Agilent). Final concentrations were calculated from a standard curve for each sphingolipid run in parallel.

Reagents

Cell and tissue sphingolipid analyses.

Cells lines were incubated in six-well tissue culture plates. Almost confluent (>90%) monolayers of cells were washed with PBS and exposed to the drugs (0.1% DMSO, myriocin, GlyH and fenretinide) dissolved in 2 ml of 50% DMEM (high glucose and L-glutamine), 50% F-12K, 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin for human airway smooth muscle cell line and DMEM (high glucose and L-glutamine), 10% FBS and 1% penicillin-streptomycin for A549 cell line, for 5 hours. After 5 hours of incubation time, the cell plates were washed extensively with PBS. 1 ml was added in each well and cells were scraped. Cell suspension was collected and centrifuged at 3000 rpm for 3 to 5 min. Cells were resuspended in 100 ul PBS, 20ul was used for protein quantification. Protein content was quantified in the washed cells by a colorimetric assay (DC Protein Assay, Bio-Rad Laboratories), and sphingolipids were quantified by high pressure liquid chromatography electrospray ionization tandem mass spectrometry (HPLC-MS/MS).

Lungs were harvested following perfusion with PBS via the right ventricle to remove blood cells from the pulmonary circulation and then homogenized. Sphinganine, sphingosine, sphinganine-1-phosphate, sphingosine-1-phosphate, ceramides (Cer 14, Cer16, Cer18, Cer20, Cer22, Cer24:1, Cer24) were quantified in lung homogenates by HPLC- MS/MS.

To avaluate altered *de novo* sphingolipid synthesis on agonist-induced intracellular calcium concentration ($[Ca^{2+}]_i$), human airway smooth muscle cells were incubated with GlyH (2 μM), fenretinide (5 μM), myriocin (1 μM), or DMSO (0.1%, control) for 5 h. Cells were then loaded with the ratiometric $[Ca^{2+}]_i$ indicator Fura2 for 40 min at room temperature and then stimulated with bradykinin (10 μM). $[Ca^{2+}]_i$ was quantified by continuously recording fluorescence using alternate excitation at 340 and 380 nm and emission at 510 nm in a microplate reader (Flexstation, Molecular Devices).

Mouse studies.

All animal studies were conducted under protocols approved by the Institutional Animal Care and Use Committee of Weill Cornell Medical College. Female C57Bl/6 mice, obtained from Taconic Farms were housed under specific pathogen-free conditions and used at 10 to 14 wk of age. GlyH-101 was freshly prepared from a 10 mM stock solution in 0.9% saline and 0.05% fatty acid free bovine serum albumin and 50 μl were administered intratracheally. Control animals received the vehicle solution (1% DMSO in 0.9% saline, 0.5% BSA) or were naive. Four hours later the animals underwent pulmonary function testing and were sacrificed. Heterozygous SPT-deficient mice ($Sptlc2^{+/-}$) or homozygous controls ($Sptlc2^{+/+}$) were bred, identified by genotyping (E2) and used at 10-14 weeks of age.

Heterozygous SPT-deficient mice or homozygous controls ($Sptlc2^{+/+}$, "WT"), originally provided by Xian-Cheng Jiang, State University of New York (SUNY) Downstate Medical Center, were bred and genotyped as described (E2).

Evaluation of lung mechanics and large airway contractility in bronchial rings

Airway reactivity *in vivo* was assessed in mice 4 h following intratracheal administration of GlyH-101 (80 μg/kg). Mice were anaesthetized with pentobarbital (100 mg/kg; American

Pharmaceutical Partners), tracheostomized and mechanically ventilated using a computer-controlled animal ventilator (Scireq). Respiratory mechanics were analyzed using the Flexivent software as previously described(E3). Broadband forced oscillations were applied to determine Newtonian (airway) resistance (Rn) using a constant phase model. Rn was also assessed following increasing nebulized doses of methacholine (3.125, 12.5 and 50 mg/ml).

To directly assess large airway reactivity murine bronchi were dissected and following removal of adhering fat and connective tissues four to eight rings of the same bronchus were prepared and mounted on hooks in a multiwire myograph system (DMT, Ann Arbor, MI) where they were kept at a resting tension of 200-250 mg in Krebs Henseleit (KH) solution containing 0.01% BSA, bubbled with 95% O_2 and 5% CO_2 at 37^{O}C and then washed every 15 min for 1 h. Mechanical forces were measured isometrically with force transducers interfaced to a data acquisition system (ADInstruments) and analyzed with LabChart6Pro for Windows. Potassium (K^+, 80 mM) was used to achieve maximum contraction and methacholine (0.01-10 μM) was added 30 min after washout of the K^+ solution. Contractile responses were measured as absolute force generated in Millinewton [mN] per mg tissue.

Evaluation of small airway contractility in precision cut lung slices (PCLS)

Mice were euthanized by intraperitoneal injection pentobarbital (100 mg/kg). The trachea was cannulated with an intravenous catheter tube (20G Intima; Becton Dickinson), connected to a catheter extension and a 4-way stopcock. A syringe filled with 3 ml of air was attached to one port while the other port was closed. The chest cavity was opened by cutting along the sternum and the ribs adjacent to the diaphragm. An agarose solution was made by dissolving and melting 0.2g of low gelling temperature agarose in 5 ml of de-ionized H_2O heated at 95ºC and

subsequently adding 5ml of 2x Hanks' balanced salt solution (HBSS) supplemented with 20 mM HEPES buffer and adjusted to pH 7.40 (sHBSS). The solution was kept at 39ºC until used for infusion. A syringe filled with the warm agarose solution (2% w/v in sHBSS) was attached to the second port of the catheter. The IV tube was clamped proximal to the trachea and purged of air with the agarose solution by allowing the trapped air to escape via a 27G needle inserted into the IV tube proximal to the clamp. The IV clamp was removed, and the lungs were re-inflated by injecting ~1.3 ml of the agarose solution. Subsequently, ~0.2 ml of air was injected into the airways to flush the agarose out of the airways and into the distal alveolar space. Immediately after agarose inflation, the lungs were soaked with ice-cold sHBSS, and the body of the animal was placed at 4°C for 15 min. The lung and heart were removed and placed in sHBSS (4°C) and cooled for an additional 30 min to ensure the complete gelling of the agarose.

To prepare PCLS, a single lung lobe was removed from the respiratory tree by cutting the main bronchus. The lung lobe was trimmed near the bronchus to produce a flat surface that adhered to the mounting block of a VF-300 vibratome (Precisionary Instruments, Greenville, NC) following manufacturer instructions. Serial lung slices (~130 μm thick) were collected in sHBSS maintained at 4°C, transferred to Petri dishes containing in DMEM supplemented with 3.7 g/L $NaHCO_3$ and an antibiotic-antimytotic mixture (ThermoFisher), and then transferred to a cell culture incubator maintained at 37°C in a humidified environment with 10% CO_2. Long-term treatment of PCLS with myriocin, GlyH-101, fenretinide, or DMSO was performed by incubating the freshly prepared PCLS for 15 hours in culture medium containing the drugs diluted at their final concentrations. After overnight incubation in culture medium, the PCLS were transferred to Hanks' balanced salt solution (HBSS) supplemented with 20 mM HEPES and adjusted to pH 7.40 (sHBSS).

PLCS containing airways that completely attached to the surrounding parenchyma and with ciliary activity were selected and mounted into a custom-made perfusion chamber consisting of a Plexiglas support for a 45 × 50 mm cover glass. A PCLS was placed in the center of the cover glass and held in place by placing a nylon mesh with a small hole in the center on the top of the PCLS. This small hole was centered on the airway of interest to allow imaging and recording of contractile activity without the interference of the mesh. A second custom-cut (11 × 40 mm) coverglass edged with silicone grease (Valve sealant; Dow Corning Co.) was placed over the slice and nylon mesh. We selected airways with an average cross-section size of 150-300 μm and confirmed that there was no significant difference in basal (un-stimulated) airway size among the testing groups ($p > 0.05$, t-test).

Perfusion of the PCLS in the chamber was performed by applying a gravity-fed flow of solution at one end of the glass chamber and suction at the other end of the chamber. The solutions perfusion was controlled by using a custom-built perfusion system consisting of eight solution reservoirs (30 ml) connected to a manifold with a single output tube (Warner Instruments Inc.). The flow from each reservoir was regulated by a valve (LFVA; Lee Company) under TTL control generated by a custom-made electronic control interfaced with the image acquisition software (Video Savant, IO Industries). The lung slices were observed on an inverted microscope (Diaphot 300; Nikon) with a 20× objective. Images were recorded with a CCD camera using a frame grabber PC card (Picolo; Eurosys) and the image acquisition software (Video Savant; IO industries, Inc.). Digital images (648 × 484 pixels) were recorded in time-lapse (0.5 Hz) and stored directly on a hard drive. The cross-sectional lumen area of the airway in each image was obtained by pixel summing using a custom-made script that runs in Video

Savant. The airway lumen area was normalized to the initial area, i.e. before the perfusion of any agonist or drug, and the changes in lumen area over time were plotted.

Methacholine (MCh) was dissolved in de-ionized H_2O to make a stock solution and dissolved in the sHBSS to its final concentration on the same day of the experiments. To evaluate airway hyperreactivity in response to increasing doses of MCh, PCLS were exposed initially to sHBSS for 5 min, followed infusion of solutions with increasing doses of MCh (0.01, 0.03, 0.1, 0.3, 1.0, 3.0, 10 μM). Each concentration was superfused for 5 min before changing to the next one, followed by a final washout with sHBSS. Solution changes in the perfusion chamber were performed according to a pre-programed time schedule and using a customized and computer-controlled perfusion system coupled to the imaging setup. Changes in airway lumen area over time were calculated by pixel summing using customized macros that run in Video Savant software.

A short incubation time of PCLS with GlyH-101 for 15 min was completed due to concerns of acute chloride channel blockage on airway reactivity. Short incubation was not needed for Fenretinide experiments.

Supplemental References

E1. Bui HH, Leohr JK, Kuo MS. Analysis of sphingolipids in extracted human plasma using liquid chromatography electrospray ionization tandem mass spectrometry. *Anal Biochem* 2012; 423: 187-194.

E2. Hojjati MR, Li Z, Jiang XC. Serine palmitoyl-CoA transferase (SPT) deficiency and sphingolipid levels in mice. *Biochim Biophys Acta* 2005; 1737: 44-51.

E3. Worgall TS, Veerappan A, Sung B, Kim BI, Weiner E, Bholah R, Silver RB, Jiang XC, Worgall S. Impaired sphingolipid synthesis in the respiratory tract induces airway hyperreactivity. *Sci Transl Med* 2013; 5: 186ra167.

Supplemental Figures and Figure Legends

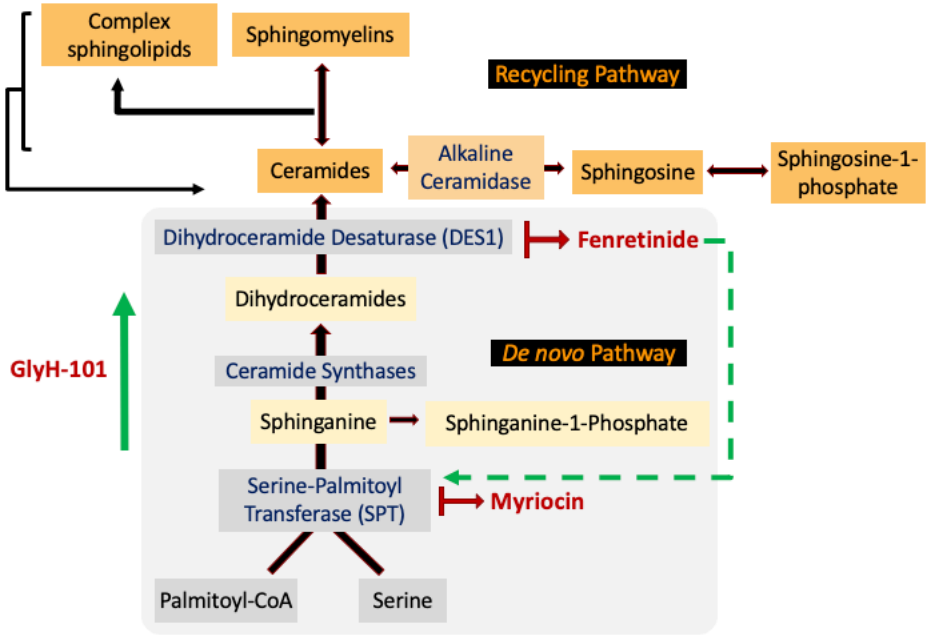

Figure E1. Sphingolipid synthesis pathways and drugs modulation. Shown diagram of the *de novo* and recycling pathways with the site of action of the drugs. SPT is inhibited by Myriocin. GlyH-101 stimulates *de novo* sphingolipid synthesis. Fenretinide inhibits dihydroceramide desaturase (DES1), resulting in a compensatory activation of SPT.

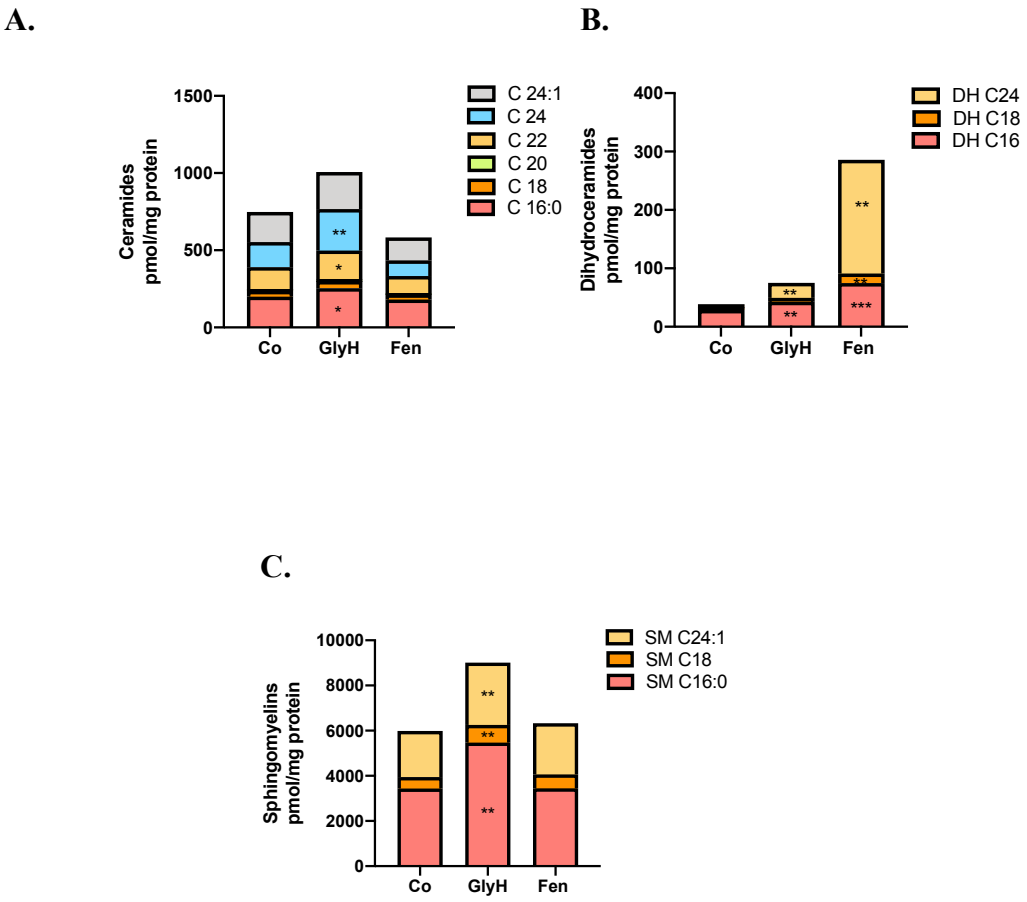

Figure E2. GlyH-101 and Fenretinide increase sphingolipids originating from de novo synthesis in A549 cells. **A.** Dihydroceramides **B.** ceramides and **C.** sphingomyelins in cultures of A549 cells treated with 2 μM GlyH-101, 5 μM fenretinide or 0.1% DMSO (control) for 5 h. Differences are compared to control group. * $p<0.05$, ** $p<0.01$, *** $p<0.001$; Kruskal-Wallis test, multiple comparisons.

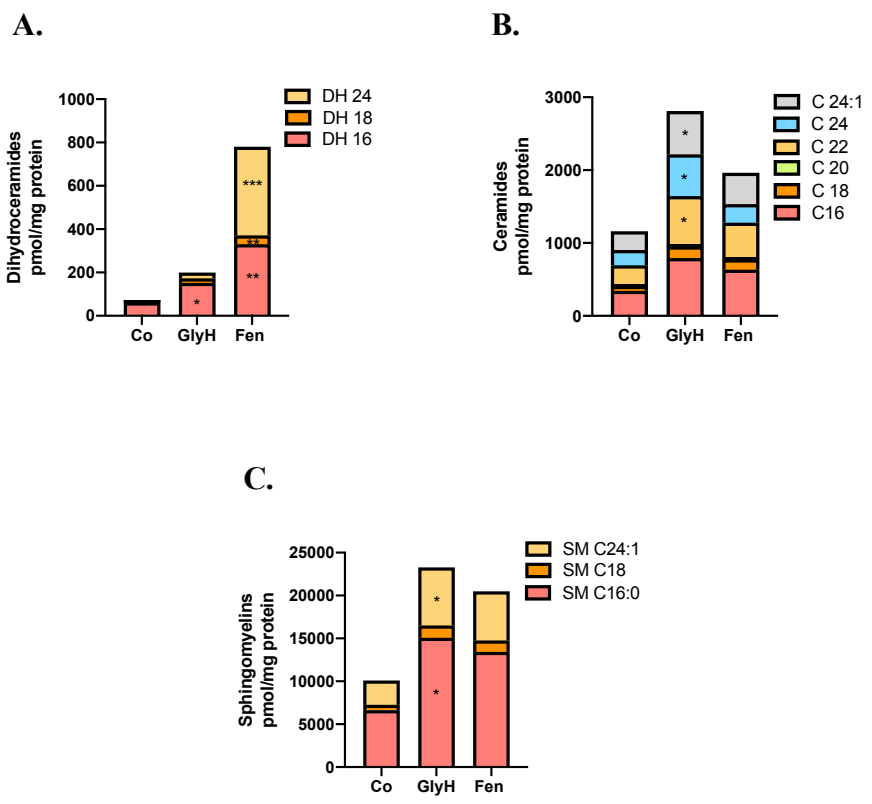

Figure E3. GlyH-101 and Fenretinide increase sphingolipids originating from de novo synthesis in human airway smooth muscle cell line. **A** Dihydroceramides, **B.** ceramides and **C.** sphingomyelins in cultures of human airway smooth muscle cells treated with 2 μM GlyH-101, 5 μM fenretinide or 0.1% DMSO (control) for 5 h. Differences are compared to control group. * $p<0.05$, ** $p<0.01$, *** $p<0.001$; Kruskal-Wallis test, multiple comparisons.

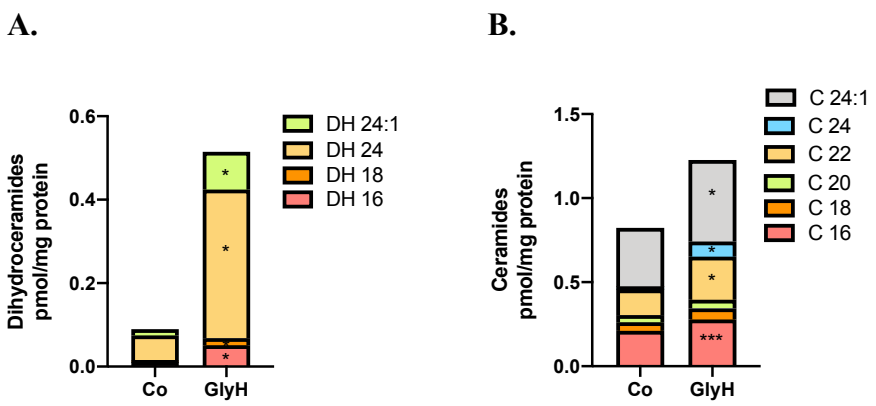

Figure E4. Sphingolipid composition in PCLS from Sptlc$^{+/-}$ mice treated with GlyH-101 for 15 h. **A** Dihydroceramides and **B.** ceramides in PCLS treated with 2 µM GlyH-101 or 0.1% DMSO (control) for 15 h. Data are means ± SEM of 52 – 58 PCLS from 4 mice in each group; * $p<0.05$, *** $p<0.001$; Mann Whitney test.

A.

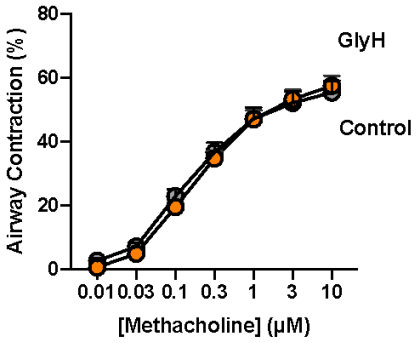

Figure E5. A. GlyH-101 does not affect MCh-induced constriction peripheral airways of WT mice. MCh induces airway contraction in PCLS from WT mice exposed to 2 µM GlyH-101 or 0.1% DMSO (control) for 15 hr. Data are means ± SEM of 36 -39 airways from 4 mice in each; Mann Whitney test.

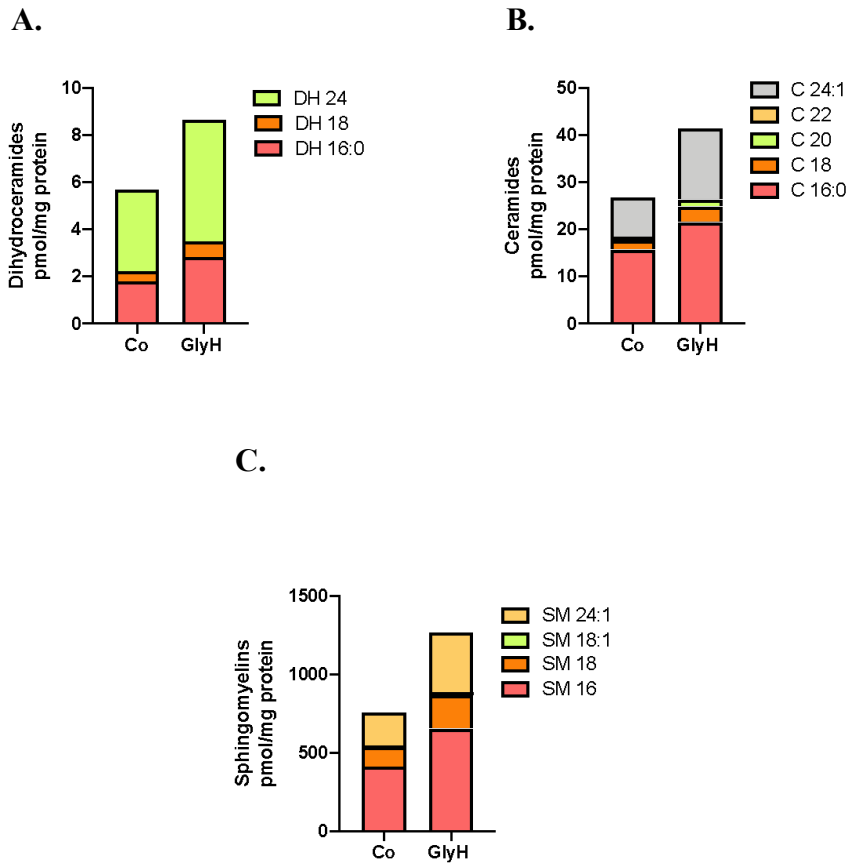

Figure E6. GlyH-101 does not affect sphingolipid composition in PCLS treated for 15 min. **A.** Dihydroceramides, **B.** ceramides and **C.** sphingomyelins in PCLS treated with 2 μM GlyH-101 or 0.1% DMSO (control) for 15 min. Data are means ± SEM of 52 – 58 PCLS from 4 mice in each group from 4 mice in each group; Mann Whitney test.

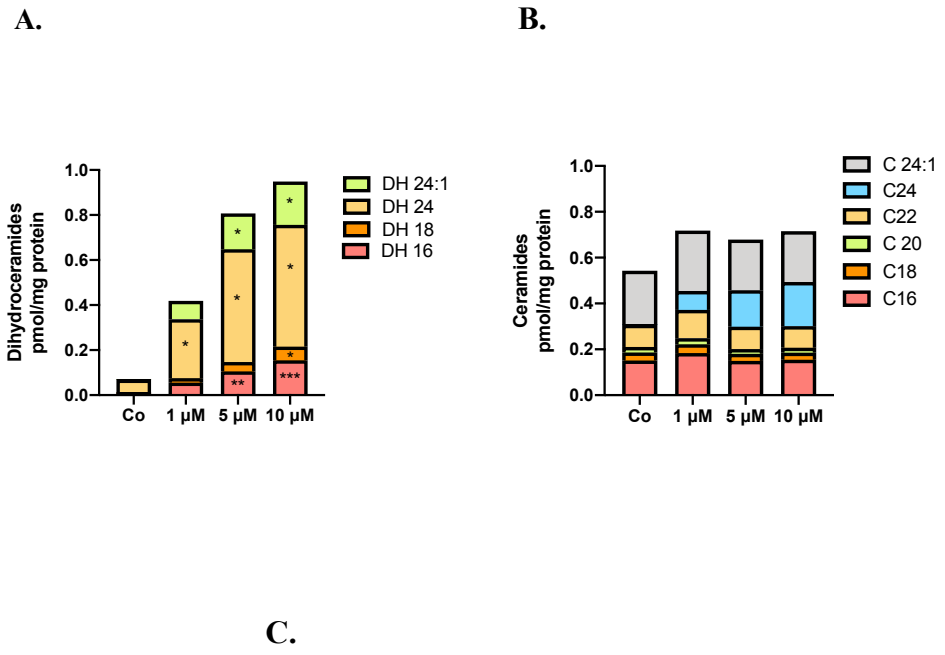

Figure E7. Fenretinide increases dihydroceramides through *de-novo* sphingolipid synthesis in PCLS. **A.** Dihydroceramides **B.** ceramides and **C.** sphingomyelins in PCLS from SPT mice and incubated with 0.1% DMSO (control) or 1, 5 and 10 µM Fenretinide for 15 h. Data are means ± SEM of 48 PCLS from 4 mice in each group; * $p<0.05$, ** $p<0.01$, *** $p<0.001$; Kruskal-Wallis test, multiple comparisons.