**Elevated Nuclear Sphingoid Base-1-Phosphates and Decreased Histone Deacetylase Activity
after Fumonisin B1 Treatment in Mouse Embryonic Fibroblasts**

Nicole M. Gardner^{a*}, Ronald T. Riley^b, Jency L. Showker^b, Kenneth A. Voss^b, Andrew J. Sachs^a, Joyce R.
Maddox^a, and Janee B. Gelineau-van Waes^a

^aDepartment of Pharmacology, Creighton University School of Medicine, Omaha, NE 68178

^bUSDA-ARS, Toxicology and Mycotoxin Research Unit, Athens, GA 30605

***Corresponding Author: Nicole M. Gardner, Department of Pharmacology, Creighton University
School of Medicine, 2500 California Plaza Omaha, NE, 68178; Tel (402) 280-3459; Fax (402) 280-
2142; E-mail: nicolegardner@creighton.edu**

ABSTRACT

Fumonisin B1 (FB1) is a mycotoxin produced by a common fungal contaminant of corn. Administration of FB1 to pregnant LM/Bc mice induces exencephaly in embryos, and ingestion of FB1-contaminated food during early pregnancy is associated with increased risk for neural tube defects (NTDs) in humans. FB1 inhibits ceramide synthase enzymes in sphingolipid biosynthesis, causing sphinganine (Sa) and bioactive sphinganine-1-phosphate (Sa1P) accumulation in blood, cells, and tissues. Sphingosine kinases (Sphk) phosphorylate Sa to form Sa1P. Upon activation, Sphk1 associates primarily with the plasma membrane, while Sphk2 is found predominantly in the nucleus. In cells over-expressing Sphk2, accumulation of Sa1P in the nuclear compartment inhibits histone deacetylase (HDAC) activity, causing increased acetylation of histone lysine residues. In this study, FB1 treatment in LM/Bc mouse embryonic fibroblasts (MEFs) resulted in significant accumulation of Sa1P in nuclear extracts relative to cytoplasmic extracts. Elevated nuclear Sa1P corresponded to decreased histone deacetylase (HDAC) activity and increased histone acetylation at H2BK12, H3K9, H3K18, and H3K23. Treatment of LM/Bc MEFs with a selective Sphk1 inhibitor, PF-543, or with ABC294640, a selective Sphk2 inhibitor, significantly reduced nuclear Sa1P accumulation after FB1, although Sa1P levels remained significantly increased relative to basal levels. Concurrent treatment with both PF-543 and ABC294640 prevented nuclear accumulation of Sa1P in response to FB1. Other HDAC inhibitors are known to cause NTDs, so these results suggest that FB1-induced disruption of sphingolipid metabolism leading to nuclear Sa1P accumulation, HDAC inhibition, and histone hyperacetylation is a potential mechanism for FB1-induced NTDs.

Keywords: Fumonisin B1; histone deacetylase (HDAC); histone acetylation; neural tube defect (NTD); sphinganine-1-phosphate (Sa1P)

53   **Abbreviations**

54   ABC, ATP binding cassette; CerS, ceramide synthase; ELEM, equine leukoencephalomalacia; FB1,
55   fumonisin B1; Gapdh, Glyceraldehyde 3-phosphate dehydrogenase; GPCR, G protein-coupled receptor;
56   HDAC, histone deacetylase; Lpp, lipid phosphate phosphatase; MEF, mouse embryonic fibroblast;
57   Na₃VO₄, sodium orthovanadate; NaF, sodium fluoride; NES, nuclear export signal; NLS, nuclear
58   localization signal; NTD, neural tube defect; PDGF, platelet-derived growth factor; PMSF,
59   phenylmethanesulfonyl fluoride; PPE, porcine pulmonary edema; PTM, post-translational modification;
60   S1P, sphingosine-1-phosphate; Sa, sphinganine; Sa1P, sphinganine-1-phosphate; SFME, serum free
61   mouse embryonic; Sgpl, sphingosine-1-phosphate lyase; Sgpp, sphingosine-1-phosphate phosphatase;
62   Sphk, sphingosine kinase; Spns2, spinster 2; So, sphingosine; TBP, TATA-binding protein; TSA,
63   trichostatin A; VPA, valproic acid

Introduction

Fumonisin B1 (FB1) is a mycotoxin produced by *Fusarium verticillioides* (previously *F. moniliforme*), a common fungal contaminant of maize (Gelderblom et al., 1988; Marasas et al., 2004). FB1 has been found in maize and maize-based foods and feed worldwide, including in the United States (Marasas, 1995; Marasas, 2001). Exposure to FB1 has been shown to cause equine leukoencephalomalacia (ELEM) (Marasas et al., 1988; Wilson et al., 1990), porcine pulmonary edema (PPE) (Harrison et al., 1990), and hepatotoxicity, nephrotoxicity, and liver and kidney carcinomas in laboratory rodents (International Agency for Research on Cancer, 2002; International Programme on Chemical Safety, 2000). Human populations that consume large amounts of FB1-contaminated foods (maize-based foods) have demonstrated a higher incidence of esophageal (Chu and Li, 1994) and liver cancer (Sun et al., 2007), and ingestion during early pregnancy is associated with increased risk of having a child with a neural tube defect (NTD) (Hendricks, 1999; Marasas et al., 2004).

NTDs (i.e. spina bifida and anencephaly) are one of the most common types of birth defect, and occur within the first month of gestation when the developing embryonic neural tube fails to close properly. The worldwide NTD average is approximately 1 in 1,000 live births; however, in regions of the world where maize is a primary food source (parts of China, Guatemala, Mexico, South Africa), the incidence of NTDs is often 6 to 11 times higher than the global average (Botto et al., 1999; Imhoff-Kunsch et al., 2007; Li et al., 2006; Marasas et al., 2004; Ncayiyana, 1986). The causes for NTDs vary greatly and are thought to be multifactorial, resulting from complex interactions between genetics, maternal nutrition, and environmental factors (Green et al., 2009). The ability of FB1 to interfere with folate transport and sphingolipid metabolism have been suggested as possible mechanisms linking FB1 exposure to human NTDs (Bulder et al., 2012; Marasas et al., 2004; Wilde et al., 2014).

FB1 has a chemical structure that is similar to that of the sphingoid bases deoxysphinganine (Zitomer et al., 2009), resulting in inhibition of ceramide synthases (CerS1-6), key enzymes involved in sphingolipid biosynthesis (Wang et al., 1991). This dysregulation of both the *de novo* and recycling

pathways of sphingolipid biosynthesis (**Fig. 1**), cause an accumulation of the sphingoid bases, sphinganine (Sa) and sphingosine (So), and their phosphorylated metabolites, sphinganine-1-phosphate (Sa1P) (also known as dihydro-S1P) and sphingosine-1-phosphate (S1P) (Gelineau-van Waes et al., 2009; Merrill et al., 2001; Zitomer et al., 2009). Sphinganine and sphingosine are phosphorylated by sphingosine kinase (Sphk) enzymes to form Sa1P and S1P, respectively. Sa1P and S1P are bioactive signaling molecules that act as ligands for a group of five G protein-coupled receptors (GPCRs), known as sphingosine-1-phosphate (S1P₁₋₅) receptors (Brinkmann, 2007; Callihan et al., 2012; Spiegel and Milstien, 2002). S1P receptors are found throughout the body and are involved in regulating a wide range of biological processes (Brinkmann, 2007; Rosen et al., 2009), and play a crucial role in embryonic development as regulators and mediators of neurogenesis and angiogenesis (Kono et al., 2004; Mizugishi et al., 2005).

There are two isoforms of Sphks, Sphk1 and Sphk2. These enzymes are largely homologous, sharing 80% sequence similarity, and are conserved across multiple species (Hait et al., 2006; Taha et al., 2006). Sphk1 and Sphk2 appear to have some redundant physiological functions as Sphk1- or Sphk2-null mice alone demonstrate reduced Sphk activity but appear to be viable, fertile, and lacking any abnormalities or malformations (Mizugishi et al., 2005). However, combined loss of both kinases results in embryos with severe abnormalities, including exencephaly (Mizugishi et al., 2005). Although similar, the subcellular localization of Sphk1 and Sphk2, as well as tissue distribution (Blondeau et al., 2007; Fukuda et al., 2003; Liu et al., 2000), are different. Sphk1 is predominantly cytoplasmic, moving to the plasma membrane upon activation, and stimulating DNA synthesis (Hengst et al., 2009; Igarashi et al., 2003; Inagaki et al., 2003; Olivera et al., 1999). Sphk2, however, is predominantly associated with the nucleus and causes inhibition of DNA synthesis and cell cycle arrest (Igarashi et al., 2003; Maceyka et al., 2005). Sphk1 is thought to be responsible for regulating levels of cytoplasmic and extracellular sphingoid base-1-phosphates (Sa1P and S1P) (Spiegel and Milstien, 2003), whereas Sphk2 is thought to be responsible for generating nuclear Sa1P and S1P (Hait et al., 2009; Riccio, 2010; Spiegel et al., 2012).

Although it is widely thought that Sphk1 and Sphk2 have distinct subcellular localizations, these appear to be dependent on cell type and density (Hait et al., 2005; Igarashi et al., 2003).

FB1-induced NTDs have previously been studied using cultured mouse embryos (Sadler et al., 2002) and, more recently, using an *in vivo* mouse model (Gelineau-van Waes et al., 2005). Mouse neurulation begins at E7.5 and is usually complete by E9.5, with these two days representing the crucial window of neural tube closure. In the inbred LM/Bc mouse strain, maternal FB1 exposure during early gestation (E7.5 – E8.5) results in a dose-dependent increase in the number of embryos with exencephaly (Gelineau-van Waes et al., 2005). At the highest dose of FB1 administered (20 mg/kg body weight/day), 79% of the LM/Bc embryos were affected with an NTD. In contrast, this dose induced less than 1% NTDs and a significant increase in the number of resorptions in the inbred SWV mouse strain (Gelineau-van Waes et al., 2005; Gelineau-van Waes et al., 2009); suggesting that genetic background plays a role in susceptibility to NTDs and/or embryonic lethality following maternal exposure to FB1. Blood spots and plasma collected from LM/Bc and SWV mice treated with FB1 demonstrate a significant elevation in sphinganine and Sa1P, suggesting that FB1 effectively inhibits CerS in the *de novo* pathway of sphingolipid biosynthesis (Gelineau-van Waes et al., 2012). Significantly higher accumulation of Sa, So and their corresponding 1-phosphates in the LM/Bc strain, compared to the SWV strain, may play a role in their susceptibility to FB1-induced NTDs (Gelineau-van Waes et al., 2012). Similar strain-specific alterations in sphingolipid metabolism have also been observed in LM/Bc and SWV strain-specific mouse embryonic fibroblasts (MEFs) exposed to FB1 (Gelineau-van Waes et al., 2012). However, in that previous study, the analysis in MEFs was done using a whole cell lysis buffer and therefore did not differentiate between Sa1P accumulation in the nuclear or cytoplasmic fractions in response to FB1 (Gelineau-van Waes et al., 2012).

We previously demonstrated that administration of the S1P receptor agonist FTY720 to pregnant LM/Bc and SWV mice results in significant accumulation of the active ligand FTY720-P in embryonic tissue and the induction of NTDs in offspring from both strains (Gelineau-van Waes et al., 2012). These results demonstrate ‘proof-of-concept’ that elevated levels of the bioactive ligand FTY720-P, a known

S1P receptor agonist and/or functional antagonist is associated with NTDs in mice. Aberrant and/or sustained activation of S1P receptors by FTY720-P during early embryonic development may play a role in the failure of neural tube closure, and these findings could implicate a potential (similar) role for elevated S1P and altered S1P receptor-mediated signaling in FB1-induced NTDs. Most research regarding sphingoid base-1-phosphates has focused on their role as S1P receptor ligands; however, recent studies have shown that Sphk2-generated S1P /S1P and FTY720-P can bind to the active sites of histone deacetylases 1 and 2 (HDAC1, HDAC2), and act as endogenous inhibitors of these HDAC enzymes, leading to increased histone acetylation (Hait et al., 2009; Hait et al., 2014). Gestational exposure to the known HDAC inhibitors valproic acid (VPA) and trichostatin A (TSA) have been shown to cause NTDs in mice and/or humans (Svensson et al., 1998; Wiltse, 2005).

The mechanism(s) underlying FB1 exposure and failure of neural tube closure are undoubtedly complex, as numerous changes occur in the balance of sphingolipid metabolites (both up- and downstream of ceramide synthase) (Gelineau-van Waes et al., 2012; Marasas et al., 2004; Merrill et al., 1993) and disruptions in folate uptake and transport (Gelineau-van Waes et al., 2005; Marasas et al., 2004; Stevens and Tang, 1997). However, in order to determine whether key mechanisms for consideration could include cytoplasmic/extracellular S1P accumulation and activation of S1P receptors, and/or nuclear accumulation of S1P and HDAC inhibition, it is necessary to identify the specific subcellular compartment in which S1P accumulates in response to FB1. The purpose of this study was to determine the levels of S1P (and S1P) that accumulate in nuclear and cytoplasmic extracts after FB1 treatment in LM/Bc and SWV MEFs and to evaluate the effects of FB1 treatment on HDAC activity and histone acetylation. Further studies utilized specific Sphk1 and/or Sphk2 inhibitors in an attempt to selectively reduce cytoplasmic vs. nuclear S1P accumulation in response to FB1. Histone post-translational modifications (PTMs) are epigenetic modifications that affect chromatin remodeling, and have the potential to alter gene regulation. Altered gene expression during the critical window of neurulation can lead to disruption of crucial, time-sensitive signaling pathways important for proper

neural tube closure. Establishing a role for cytoplasmic and/or nuclear accumulation of Sa1P in response to FB1 could provide significant insight into the potential mechanism of FB1-induced NTDs.

Materials and Methods

Mouse Embryonic Fibroblast (MEF) Cell Lines and Treatments

Strain-specific primary MEF cell lines were generated from gestational day 12.5 embryos harvested from untreated SWV and LM/Bc dams and cultured using standard conditions as previously described (Gelineau-van Waes et al., 2012). Primary fibroblasts were routinely passaged every 3 days through initial growth and senescence. Spontaneous immortalization was achieved after more than 20 passages (Gelineau-van Waes et al., 2012). For experiments, spontaneously immortalized MEFs were cultured in 100-mm tissue culture dishes for 48 hours followed by a change of media (containing serum) and treatment. MEFs were treated with vehicle (sterile water; 1.4% final concentration) or 40 μ M FB1 (Cayman Chemical Co., Ann Arbor, MI) and incubated for an additional 24 hours. For experiments utilizing PF-543 (Millipore, Billerica, MA), a selective Sphk1 inhibitor, MEFs were grown for 48 hours, then simultaneously treated with vehicle (DMSO; 0.1% final concentration) or 1 μ M PF-543 and 40 μ M FB1 at the time of media change. PF-543 treatment duration and dose were determined based on previous literature (Lynch, 2012; Schnute et al., 2012) and small concentration-response studies (data not shown) conducted in our laboratory. For experiments utilizing ABC294640 (MedKoo Biosciences, Chapel Hill, NC), a selective Sphk2 inhibitor, at the time of media change, MEFs were treated with vehicle (DMSO; 0.2% final concentration) or 50 μ M ABC294640 and incubated for 24 hours. Following the 24 hour pre-treatment with ABC294640, the MEFs were treated with vehicle (sterile water) or 40 μ M FB1 for another 24 hours. ABC294640 treatment duration and dose were also determined based on small concentration-response studies (data not shown) and published data (French et al., 2010; Gao et al., 2012). When both inhibitors were used, MEFs were treated with PF-543, ABC294640, and FB1 at the time of media change, and cells were allowed to grow for 24 hours. Treatments did not appear to have a

significant effect on cell viability, as MEFs continued to replicate and actively grow throughout the entire treatment period.

Isolation of Nuclear and Cytoplasmic Fractions (NE-PER kit)

The nuclear and cytoplasmic cell fractions were isolated from control and treated MEFs for analysis of free sphingoid bases and sphingoid base-1-phosphates using a slight modification of the NE-PER kit (78835, Thermo Scientific, Waltham, MA) manufacturer's protocol. Reagent volumes were based on a packed cell volume of 10 μ L: 100 μ L of CER I, 5.5 μ L of CER II, and 50 μ L of NER. CER I and NER also contained Halt™ Protease Inhibitor Cocktail (78425, Thermo Scientific), as well as sodium orthovanadate (Na_3VO_4) at 1 mM, sodium fluoride (NaF) at 10 mM, and phenylmethanesulfonyl fluoride (PMSF) at 0.5 mM. After the cytoplasmic and nuclear fractions were collected, a 96-well plate Bradford assay (Sigma, B6916) was performed using a SpectraMax M3 microplate reader (Molecular Devices, Sunnyvale, CA) to determine the protein concentration of each cell fraction compared to a standard curve generated using bovine serum albumin (BSA). Aliquots of 100-200 μ g of nuclear and 400-600 μ g cytoplasmic cell fractions were frozen at -80°C until further analysis of sphingoid bases and sphingoid base-1-phosphates by high performance liquid chromatography tandem linear-ion trap electrospray ionization (LC-ESI-MS/MS). The integrity of the cytoplasmic and nuclear fractionation has been previously examined multiple times through the use of cytoplasmic (acetylated tubulin or Glyceraldehyde 3-phosphate dehydrogenase (Gapdh) and nuclear (lamin B1 or TATA-binding protein (TBP)) localized proteins as loading controls for other experiments not presented here (data not shown).

Mass Spectrometry Analyses of Sphingolipids

Free sphingoid bases and sphingoid base 1-phosphates were quantitated by LC-ESI-MS/MS using a modification of the method of Zitomer et al. (2008, 2009). Briefly, the cells were lysed using the NE-PER kits and analyzed for Bradford protein as described above. Aliquots of the nuclear fraction (100-200 μ g protein in 40 μ L of nuclear lysate) and the cytoplasmic fraction (400-600 μ g protein in 90 μ L of cell lysate) were transferred to 1.5 mL polypropylene tubes. The extraction mix for both the nuclear and

cytoplasmic aliquots contained formic acid, water, acetonitrile, and the sphingolipid standards at concentrations such that when added to the polypropylene tubes containing the nuclear or cytoplasmic lysates the final volume would be 600 μ l and the final concentrations would be 50% acetonitrile, 45% water, and 5% formic acid and a total of 36 pmol of C20-dihydrosphingosine (C20-Sa, d20:0) (Matreya, Pleasant Gap, PA) and D-erythro-C17-sphingosine-1-phosphate (C17-S1P) (Avanti Polar Lipids, Alabaster, AL) as standards in each tube of the cytoplasmic or nuclear lysate. The recovery of C17-S1P and C20-Sa from nuclear cell lysates to which known amounts of C17-S1P and C20-Sa had been added was 91.4% $\pm$ 0.02% (mean $\pm$ SD, n=5) and 99.3% $\pm$ 0.02% (mean $\pm$ SD, n=5), respectively. The recovery of C17-S1P and C20-Sa from cytoplasmic cell lysates to which known amounts of C17-S1P and C20-Sa had been added was 95.9% $\pm$ 0.04% (mean $\pm$ SD, n = 5) and 95.6% $\pm$ 0.03% (mean $\pm$ SD, n=5), respectively. The recoveries of C17-S1P and C20-Sa were slightly less (82% to 94%) when lysis buffer without cells was extracted. The extracts were analyzed for C17-S1P and C20-Sa standards and for D-erythro-sphingosine 1-phosphate (S1P), D-erythro-dihydrosphingosine-1-phosphate (Sa1P), 1-deoxy-sphinganine (1-deoxy Sa, m18:0), DL-erythro-dihydrosphingosine (Sa, d18:0), and D-erythro-sphingosine (So, d18:1) (Sigma-Aldrich, St. Louis, MO). LC-ESI-MS/MS analysis of the extracts was conducted using a Finnigan Micro AS autosampler coupled to a Surveyor MS pump (Thermo Fisher Scientific, Waltham, MA). Separation was accomplished using an Imtakt Cadenza 3- μ m particle size CW-C18 column, 150 $\times$ 2 mm (Imtakt USA, Portland, OR). The flow rate was 0.2 ml/min and the initial mobile phases used were 97% acetonitrile/2% water/1% formic acid (solvent A) and 2% acetonitrile/97% water/1% formic acid (Solvent B). The gradient ran from 60% A, 40% B to 90% A, 10% B at 15 min and then ramped to 100% A at 20 min followed by 10 min at 100% A and then a 10 min re-equilibration of the column at 60% A, 40% B for a total run time of 40 min. The column effluent was directly coupled to a Finnigan LTQ linear ion trap mass spectrometer operating in the electrospray ionization (ESI) positive ion mode with an ion transfer tube temperature of 210°C and a sheath gas of nitrogen. For LC-ESI-MS/MS of sphingoid base 1-phosphates and sphingoid bases, the collision energy was 35% and 20%, respectively. Quantification of sphingoid bases and their respective 1-phosphates was accomplished by

comparing the integrated area for the known quantity of the appropriate internal or external standard to the area of the analyte. The levels of sphingoid bases and sphingoid base 1-phosphates detected in the cytoplasmic and nuclear protein extracts were assumed to be representative of in situ levels present in the nuclear and cytoplasmic compartments of intact MEFs.

Histone Deacetylase (HDAC) Activity

The nuclear cell fraction was isolated from control and treated MEFs for determination of HDAC activity using a slight modification of the HDAC Activity Assay (Cayman Chemical Co., Ann Arbor, MI) manufacturer's protocol. Lysis Buffer, resuspension buffer, and Extraction Buffer were all prepared as instructed but additional phosphatase inhibitors and a protease inhibitor were added to prevent degradation of the sphingoid base-1-phosphates. The phosphatase inhibitors used were sodium orthovanadate (Na_3VO_4) at 1 mM and sodium fluoride (NaF) at 10 mM. The additional protease inhibitor phenylmethanesulfonyl fluoride (PMSF) was also added at 0.5mM. Finally, nuclear proteins were isolated in 200 μl of Extraction Buffer and sonicated, using a Branson UltrasonicsSLPe Digital Sonifier Cell Disruptor (Branson Ultrasonics Co., Danbury, CT). Samples were placed in an ice bath and sonicated for 10 seconds (30% power), followed by a 30-second break, and then sonicated again for another 10 seconds. Protein concentration was determined by Bradford assay. HDAC activity was determined using the Cayman HDAC Activity Assay Kit (Cayman Chemical Co., 10011563, Ann Arbor, MI). Samples were diluted in extraction buffer so that all samples contained equal concentrations (0.2mg/ml) before being added to the assay. Absorbance was read on a SpectraMax 3 (Molecular Devices, Sunnyvale, CA) with SoftMax Pro 5.4.1 software using an excitation wavelength of 350nm and an emission wavelength of 453nm. Activity was calculated based on absorbance compared to a standard curve generated as part of the assay. This assay measures the activity of Class I and II HDACs.

Histone Purification

Histone Purification Mini Kits (Active Motif, 40026, Carlsbad, CA) were used to isolate and purify histones from control and FB1-treated MEFs. Protocol was followed per manufacturer's

specifications. Cells were washed twice with pre-warmed serum-free medium, 800 μ L of Extraction Buffer, was added to the cells and a cell scraper was used to remove the cells, which were then transferred to a microcentrifuge tube and placed on a rotating platform at 4°C for 2 hours. The extraction and purification protocol followed the manufacturer's protocol. The samples continued through the precipitation step (in protocol) to further concentrate the histones. The final pellet was then resuspended in 50 μ L of sterile water for western blots. A NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, ND-2000C, Waltham, MA) was used to quantify the histone yield of each sample. Using the molecular weight (MW) and extinction coefficient (E) of core histones (H2B: MW=13.8 kDa, E=6070, H3: MW=15.3 kDa, E= 4040, and H4: MW=11.2 kDa, E= 5400), the concentration of each sample was determined in μ g/ μ L.

Western Blot Analysis of Histone Post-translational Modifications

Following histone purification from control and FB1-treated MEFs (in triplicate), western blots were performed to confirm that FB1-treatment resulted in increased histone acetylation, specifically in the lysine residues predicted by Hait et al. (2009), as well as PTMs identified by LC/LC/MS/MS of serum free mouse embryonic (SFME) neural stem cells, isolated from Balb/c mice (*manuscript submitted*). Western blots were performed using Novex® 10-20% Tris-Glycine gels (Life Technologies, EC61385Box, Grand Island, NY), loading 0.5 μ g of histone proteins from each sample. The gels were transferred to Immobilon®-FL PVDF membranes (Millipore, IPFL10100, Billerica, MA) and, once transfer was complete, the membranes were placed in Odyssey® Blocking Buffer (Li-Cor, 927-40000, Lincoln, NE) for two hours. The membranes were then placed in primary antibody overnight at 4°C. Primary antibodies included: H2BK12ac (1:2000, Abcam 40883); H3K9ac (1:2000, Qiagen GAM-1209); H3K18ac (1:1000, Active Motif 39755), H3K23ac (1:1000, Active Motif 39131); unmodified H2B (1:250, Active Motif 39210); and, H3 (1:2000, Qiagen GAM-2206). A fluorescent goat anti-rabbit IRDye® 680 LT secondary (1:20,000; 926-68021, Li-Cor, Lincoln, NE) was added to each membrane for one hour

at room temperature, and protein bands were then visualized using an Odyssey 9120 (Li-Cor, Lincoln, NE) and Image Studio (Version 3.1) software.

Statistical Analysis

All experiments were done in triplicate and results are expressed as means $\pm$ standard error of the mean (SEM). For comparison of two groups, statistical significance was determined using a Student's *t*-test. Tests were two-tailed and differences were considered significant at p -value ≤ 0.05 . For comparison of multiple groups, an analysis of variance (ANOVA) was performed, followed by a Student-Newman-Keuls *post hoc* test to determine significance ($p \leq 0.05$).

Results

Elevated Sphingoid Bases and 1-Phosphates in the Nucleus of LM/Bc and SWV MEFs after FB1 Treatment

LC-ESI-MS/MS analysis of the cytoplasmic and nuclear extracts was performed to determine the subcellular localization of free sphingoid bases (Sa and So) and sphingoid base-1-phosphates (Sa1P and S1P) after FB1 treatment (**Fig. 2A-B**). Sa and Sa1P were significantly elevated in the cytoplasm and nucleus of both SWV and LM/Bc MEFs 24 hours after FB1 treatment (**Fig 3A-B**). Although the FB1-induced increase in Sa and Sa1P was observed in both fractions, the amount found in the nuclear fraction was significantly more than the amount in the cytoplasmic fraction in response to FB1 treatment across both strains. Consistently, the increase in both Sa and Sa1P were significantly higher in LM/Bc MEFs compared to SWV MEFs. Most notably, FB1-treated LM/Bc MEFs had significantly more nuclear Sa1P (1150 ± 42 pmol / mg protein, $p \leq 0.0001$) than treated SWV MEFs (45 ± 3 pmol / mg protein). The concentrations of Sa1P detected in the nuclear fraction of LM/Bc MEFs was 6-fold greater than the concentrations of Sa1P detected in the cytoplasmic fraction ($p \leq 0.0001$). FB1 treatment also resulted in an increase in sphingosine (So) within both the LM/Bc and SWV MEFs, with a greater accumulation in the nuclear fraction of LM/Bc MEFs treated with FB1 (data not shown). S1P was only detected in the LM/Bc MEFs after FB1 treatment (data not shown). The amount of nuclear Sa1P was approximately 30-

fold higher than the amount of nuclear SaIP in the LM/Bc MEFs. Repeated LC-ESI-MS/MS analysis of FB1 and control MEFs always show increased SaIP in the LM/Bc strain and differences between SaIP accumulation in the nuclear fraction relative to the cytoplasmic fraction were consistently observed, suggesting that elevated nuclear SaIP may represent a novel response to FB1.

Decreased Histone Deacetylase (HDAC) Activity in LM/Bc and SWV MEFs Treated with FB1

To test the hypothesis that elevated nuclear SaIP after FB1 treatment might act as endogenous inhibitors of HDAC activity an HDAC activity assay was performed. Basal HDAC activity in the nuclei of control LM/Bc MEFs (0.21 ± 0.0076 nmol / min / mL / μ g protein) was significantly lower than that observed in control SWV MEFs (0.31 ± 0.021 nmol / min / mL / μ g protein; $p=0.02$) (**Fig. 4**). The basal level of HDAC activity in control LM/Bc MEFs was similar to the level of HDAC activity in SWV MEFs that had been treated with FB1. In LM/Bc MEFs there was a significant decrease in nuclear HDAC activity in response to FB1 treatment (0.12 ± 0.017 nmol / min / mL / μ g protein, $p=0.014$).

Increased Histone Acetylation in LM/Bc MEFs after FB1 Treatment

Because elevated levels of nuclear SaIP and decreased HDAC activity were observed in FB1-treated LM/Bc MEFs, follow-up studies were done to examine changes in histone acetylation. Western blot analysis identified a significant increase in H2BK12ac and H3K9ac after FB1 treatment (**Fig. 5**); however H4K5ac did not increase after FB1 treatment (data not shown). FB1-treated LM/Bc MEFs also showed a significant increase in H3K23ac (**Fig.5 and inset**).

Decreased Nuclear SaIP in LM/Bc MEFs Pre-Treated with ABC294640 and FB1

Because Sphk2 is thought to be predominantly associated with the production of nuclear SaIP (Igarashi et al., 2003; Maceyka et al., 2005), a Sphk2-selective competitive inhibitor, ABC294640, was used to reduce nuclear SaIP. After FB1 treatment alone, expected nuclear SaIP accumulation was observed (606 ± 123 pmol / mg protein, $p=0.04$). Pre-treatment with ABC294640, prior to FB1 treatment, significantly

reduced the elevated nuclear SaIP levels by approximately 85% (97 ± 24 pmol / mg protein, $p=0.05$) (Fig. 6). Although the nuclear SaIP level was significantly reduced by ABC294640, it remained higher than basal levels. FB1 treatment alone caused a small increase in cytoplasmic SaIP that was not reduced by ABC294640, consistent with evidence that Sphk2 is primarily associated with the nucleus (Fig. 6).

Decreased Nuclear SaIP in LM/Bc MEFs Treated with PF-543 and FB1

To determine the role of Sphk1 in SaIP production/accumulation, a selective Sphk1 inhibitor (PF-543) was used. After FB1 treatment alone, SaIP accumulation was observed in both subcellular compartments (cytoplasmic: 163 ± 11 pmol / mg protein; nuclear: 548 ± 39 pmol / mg protein). Concurrent treatment with FB1 and PF-543 did not reduce the cytoplasmic SaIP elevation as predicted (120 ± 13 pmol / mg protein). However, PF-543 treatment did significantly reduce nuclear SaIP accumulation by approximately 35% (368 ± 31 pmol / mg protein; $p=0.02$) (Fig. 7). Suggesting that in MEFs, Sphk1 may contribute to generation of SaIP in both the nuclear and cytoplasmic compartment.

Complete Reduction in Nuclear SaIP of LM/Bc MEFs Treated with both Sphk inhibitors and FB1

Because individual treatments with PF-543 or ABC294640 were not able to reduce nuclear SaIP levels back to basal levels, simultaneous treatment with both inhibitors was tested. Concurrent PF-543 and ABC294640 treatment resulted in a significant decrease in cytoplasmic SaIP (44 ± 4.4 pmol / mg protein, $p=0.0006$); however cytoplasmic SaIP levels were still significantly elevated compared to the control LM/Bc MEFs (Fig. 8). In contrast, treatment with both inhibitors resulted in complete depletion of nuclear SaIP, suggesting that in MEFs, both Sphk1 and Sphk2 contribute to the generation of nuclear SaIP ($p=0.005$) (Fig. 8).

Discussion

The present study demonstrates that FB1 treatment of LM/Bc MEFs results in a significant accumulation of SaIP, primarily in the nuclear protein fraction. The total amount of SaIP detected in

both the nuclear and cytoplasmic fractions of LM/Bc MEFs is more than 25-times the amount detected in SWV MEFs. These results parallel the strain-specific differences in accumulation of sphingolipid metabolites in blood and tissues when pregnant LM/Bc and SWV mice are treated with FB1. The amount of nuclear Sa1P in LM/Bc MEFs is more than 5 times the amount of cytoplasmic Sa1P. We previously reported the elevation in Sa1P after FB1 treatment in whole cell lysates of MEFs (Gelineau-van Waes et al., 2012); however, this is the first report of nuclear accumulation of Sa1P after FB1 treatment. Although other sphingolipid metabolites are altered after FB1 treatment, the elevation in Sa1P, specifically nuclear Sa1P, which is approximately 30 times higher than the amount of nuclear S1P, suggests that Sa1P may play an important role in induction of biological effects of FB1 that contribute to increased risk of NTDs.

Understanding the relative importance of Sphk1 and Sphk2 in the production of Sa1P and S1P in MEFs and the specific subcellular compartment in which these metabolites accumulate is an important first step in elucidating the mechanistic role of histone acetylation and further defining the importance of sphingolipid metabolism disruption in the relative strain-specific sensitivity to FB1-induced NTDs. Hait et al. (2009) demonstrated that overexpression of Sphk2 results in increased production/accumulation of S1P and Sa1P in the nuclear compartment of breast cancer cells. They further demonstrated that nuclear S1P and Sa1P can bind and inhibit the activity of HDAC1 and HDAC2, leading to increased acetylation of histone lysine residues H2BK12, H3K9, and H4K5. The experiments presented here, in LM/Bc MEFs, revealed an increase in histone acetylation at H2BK12, H3K9, and H3K23. It was also recently shown that nuclear concentrations of S1P are elevated in MEFs derived from *Sgpl* knockout mice (Ihlefeld et al., 2012). *Sgpl* is responsible for irreversibly degrading Sa1P and S1P to phosphoethanolamine and hexadecanal (Spiegel and Milstien, 2002). *Sgpl* knockout MEFs exhibit decreased HDAC activity and increased H3K9 acetylation (Ihlefeld et al., 2012). In further support of these findings, oral administration of an *Sgpl* inhibitor, THI, in a mouse model of muscular dystrophy resulted in increased S1P and Sa1P in nuclear extracts from adductor muscles, HDAC inhibition, and increased acetylation of H2BK12, H3K9, and H3K18 (Nguyen-Tran et al., 2014).

The teratogenicity of known HDAC inhibitors has been widely studied for many years in humans and animal models. Extensive research has been done on valproic acid (VPA), an anticonvulsant used to treat epilepsy and bipolar disorder, and its HDAC inhibition. A single dose of VPA on gestational day 8 caused exencephaly in NMRImouse embryos (Kultima et al., 2004), while three doses of VPA on gestational day 9 resulted in mouse embryos with a high incidence of spina bifida occulta (Ehlers et al., 1992). VPA is also teratogenic in humans; pregnant epileptic women taking VPA during the first trimester run the risk of having a child with spina bifida (Lammer et al., 1987; Oakeshott and Hunt, 1989). Another known HDAC inhibitor that has teratogenic effects is trichostatin A (TSA), a compound originally isolated as an antifungal antibiotic (Vanhaecke et al., 2004). Similar to VPA, TSA treatment of cultured mouse embryos resulted in defects in embryo rotation and neural tube closure (Svensson et al., 1998), while *in vivo* TSA exposure resulted in many axial skeletal malformations (Menegola et al., 2005). Direct injection of TSA into the posterior neural tube of chick embryos resulted in a significant increase in neural tube closure defects and abnormalities (Murko et al., 2013). Our data demonstrate that FB1 exposure and elevated nuclear Sa1P are associated with decreased HDAC activity and increased histone acetylation. Based on the previously demonstrated link between gestational exposure to other HDAC inhibitors and failure of neural tube closure, our findings implicate involvement of a similar mechanism in FB1 teratogenicity in the LM/Bc mouse and possibly other species.

Although exposure to other known HDAC inhibitors during pregnancy has been shown to cause NTDs (Ehlers et al., 1992; Gurvich et al., 2005; Kultima et al., 2004; Lammer et al., 1987; Murko et al., 2013; Oakeshott and Hunt, 1989; Svensson et al., 1998; Wiltse, 2005), the critical histone PTMs and subsequent alterations in gene regulation that result in NTDs are not fully understood. Tung and Winn (Tung and Winn, 2010) showed an overall increase in H3 and H4 acetylation in VPA-treated exencephalic embryos, and Hezroni et al. (2011) demonstrated increased H3K9ac after VPA treatment of embryonic stem cells. Recent research by Tsurubuchi et al. (2013) has revealed potential PTMs that could act as biomarkers for early detection of human fetuses with a NTD. Stem cells were isolated from the amniotic fluid of pregnant women and stained for different epigenetic markers. Interestingly, their

results showed that H3K9ac and H3K18ac were increased in the cells collected from a woman pregnant with an anencephalic fetus, but not in those who had normal fetuses or a fetus with a myelomeningocele, suggesting that increased H3K9 and H3K18 acetylation may be an indicator of anencephaly in humans (Tsurubuchi et al., 2013). Although our results are similar to the findings of Tsurubuchi et al. (2013) (increased H3K9 and H3K18 acetylation), the significance of these particular histone PTMs and their role in gene regulation and/or relationship to NTDs is currently unknown.

Histone acetylation has long been known to cause the opening of chromatin, resulting in transcriptional activation (Selvi and Kundu, 2009) that could ultimately alter time-sensitive programs of gene regulation and activation/inhibition of signaling pathways crucial for proper development. Thus, inhibition of HDAC1 and HDAC2 may also play a role in the FB1-induced NTDs. HDAC1 is highly expressed in nearly all mouse embryonic tissues, including developing neural folds, and is present throughout embryonic development (Lagger et al., 2002). The loss of HDAC1 alone has been shown to significantly reduce total deacetylase activity, even though other HDACs are present, suggesting that HDAC1 has a large functional role. HDAC1-null embryos exhibit severe cranial abnormalities at E9.5 and do not survive past E10.5 (Lagger et al., 2002). Similarly to HDAC1, HDAC2 has also been localized to both embryonic neural stem cells and neural progenitor cells throughout development (MacDonald and Roskams, 2008). Deletion of both HDAC1 and HDAC2 in developing neurons resulted in severe cortical, hippocampal, and cerebellar abnormalities, ultimately leading to death by postnatal day 7 (Montgomery et al., 2009). Thus, HDAC1 and HDAC2 are required for proper CNS development. Our results show that FB1 treatment results in increased nuclear Sa1P and decreased HDAC activity, providing a plausible mechanistic role for HDAC inhibition in NTD development following FB1 exposure in LM/Bc mice.

Pre-treatment with ABC294640, a selective Sphk2 inhibitor, prior to FB1 treatment in LM/Bc MEFs significantly reduced the nuclear accumulation of Sa1P, without decreasing the cytoplasmic accumulation. Studies with PF-543 indicate its selectivity for Sphk1 over Sphk2 as well as other kinases. PF-543 has been reported to be over 100-times more potent inhibitor of Sphk1 versus Sphk2 (Lynch,

2012). Although Sphk1 is thought to be associated with the cytoplasm and plasma membrane (Hengst et al., 2009; Igarashi et al., 2003; Inagaki et al., 2003; Olivera et al., 1999), concurrent treatment with PF-543, a selective Sphk1 inhibitor, and FB1 resulted in a significant decrease in nuclear Sa1P but had no effect on cytoplasmic Sa1P. The translocation of Sphk1 from the cytoplasm to the nucleus was first reported in response to platelet-derived growth factor (PDGF) (Kleuser et al., 2001). Two functional nuclear export signal (NES) sequences have been found within human Sphk1, allowing this kinase to undergo nucleocytoplasmic translocation (Inagaki et al., 2003). Unfortunately, the nuclear function, as well as the importance of the nucleocytoplasmic translocation, of Sphk1 is not well known. Igarashi et al. (2003) demonstrated that the fusion of Sphk1 with the nuclear localization signal (NLS) sequence from Sphk2 resulted in nuclear Sphk1 and inhibition of DNA synthesis, whereas its cytoplasmic localization would have typically stimulated DNA synthesis, suggesting that nuclear localization is extremely important.

The results of this study demonstrate a potential novel mechanism of action for FB1-induced NTDs following ceramide synthase inhibition. The increase in nuclear Sa1P, and corresponding decrease in HDAC activity and increased histone acetylation, could result in altered epigenetic regulation of gene expression, and this in turn may contribute to the failure of neural tube closure observed in LM/Bc embryos exposed to FB1. Future studies will determine if pharmacological inhibition of Sphk2 (or knockdown of *Sphk2*) can reverse the observed HDAC inhibition and increased histone acetylation in LM/Bc MEFs. *In vivo* studies will also be conducted to determine if Sphk2 inhibition (or genetic inactivation of *Sphk2* on an LM/Bc background) is capable of reducing the number of FB1-induced NTDs in pregnant LM/Bc mice. Alternatively, the significant increase in cytoplasmic S1P, observed only in the LM/Bc MEFs, and the increased cytoplasmic Sa1P, may have implications for sustained and/or aberrant activation of S1P receptor-mediated signaling pathways in the etiology of FB1-NTDs. Further studies will be needed to separate the relative importance of bioactive S1P and Sa1P that accumulates in each cell compartment after FB1 exposure and their mechanistic role in the regulation of epigenetic and/or cell signaling pathways *in vivo* that result in NTDs.

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FIGURE 1

FIGURE 1: Sphingolipid metabolism pathways. *De novo* sphingolipid biosynthesis begins with the condensation of serine and palmitoyl-CoA by serine palmitoyltransferase (Sptlc), to form sphinganine (Sa) (other amino acids, most notably alanine and glycine can also be used by Sptlc). Sphinganine is then acylated by (dihydro) ceramide synthase enzymes (CerS1-6) to form dihydroceramide, which is reduced to form ceramide, by dihydroceramide desaturase (Des). Sphinganine can also be phosphorylated by sphingosine kinases (Sphk1, Sphk2) to form sphinganine-1-phosphate (Sa1P), a bioactive signaling molecule. Cytoplasmic Sa1P can be transported out of the cell by members of the ABC transporter family to act on cell surface G protein-coupled S1P receptors (S1Pr1-5). Nuclear Sa1P has been shown to inhibit histone deacetylases (HDACs). Sa1P can be dephosphorylated by sphingosine phosphate phosphatases (Sgpp) or by lipid phosphate phosphatases (Lpp) to form Sa. In the recycling sphingolipid pathway, ceramide can be de-acylated by ceramidase to form Sphingosine (So), which can be acylated by CerS1-6 to reform ceramide. Similar to sphinganine, sphingosine can be phosphorylated by Sphk1 and Sphk2 to form sphingosine-1-phosphate (S1P). S1P is also a bioactive signaling molecule that can act on S1P receptors or inhibit HDACs. Fumonisin exposure or treatment inhibits ceramide synthase enzymes resulting in a decrease of ceramide production and an increase in Sa, So, Sa1P, and S1P.

FIGURE 2:

FIGURE 2: Absolute intensity of C17 and Sa1P within the nuclear fraction of FB1-treated LM/Bc and SWV MEFs. Representative LC-ESI-MS/MS data from the nuclear fraction of FB1-treated LM/Bc (A) and SWV (B) MEFs, showing peaks for the parent ion of the C17 S1P (m/z 366.3) standard (36 pmol) and Sa1P (m/z 382.2) (arrows indicate appropriate peaks). These graphs demonstrate the relative difference in accumulation of Sa1P observed within the LM/Bc strain compared to the SWV strain after FB1-treatment. The first peak, at approximately 2 minutes, is a compound with a similar mass to charge ratio (m/z) of 382, but is not Sa1P due to having the wrong retention time and MS/MS mass spectra (data not shown).

FIGURE 3:

FIGURE 3: Relative accumulation of sphinganine and sphinganine-1-phosphate in the nuclear and cytoplasmic fraction of MEFs treated with FB1. Sphinganine (Sa) and sphinganine-1-phosphate (Sa1P) were analyzed by LC-ESI-MS. Data are shown as mean $\pm$ SEM ($n=3$ for each group). #The mean level of the sphingolipid analyzed in the nuclear fraction of FB1-treated cells is significantly more than the corresponding cytoplasmic fraction within the same strain ($p \leq 0.0001$). **The mean level of sphingolipid analyzed in the cytoplasmic or nuclear fraction of FB1-treated LM/Bc MEFs was significantly more than the corresponding fraction of FB1-treated SWV MEFs ($p \leq 0.001$). ‡The mean level of Sa in the cytoplasmic or nuclear fraction of FB1-treated MEFs was significantly more than that found in the corresponding fraction of control MEFs ($p \leq 0.0001$). ND signifies that the analyte was either not detected or present in an amount that could not be quantified (trace amount).

FIGURE 4:

FIGURE 4: Histone deacetylase activity in SWV and LM/Bc MEFs. Histone deacetylase (HDAC) activity was measured through an HDAC activity assay, utilizing nuclei of LM/Bc and SWV mouse embryonic fibroblasts (MEFs). Data are shown as mean HDAC activity $\pm$ SEM ($n=3$ for each group). ** The mean HDAC activity of the FB1-treated LM/Bc MEFs was significantly decreased compared to that of the control LM/Bc MEFs (** $p < 0.02$). # The mean HDAC activity level of the control LM/Bc MEFs was significantly lower than that of the control SWV MEFs ($p < 0.02$).

FIGURE 5:

FIGURE 5: Western blot analysis of increased histone acetylation in LM/Bc MEFs treated with FB1. Data are shown as mean expression compared to unmodified core histone (loading control) $\pm$ SEM (in triplicates). Inset is a visual representative of western blots performed for histone acetylation. ** The mean histone acetylation expression in LM/Bc FB1-treated MEFs was significantly higher than that found in LM/Bc control MEFs ($p < 0.03$).

FIGURE 6:

FIGURE 6: Sa1P levels after pre-treatment with ABC294640, a selective Sphk2 inhibitor, followed by FB1 treatment in LM/Bc MEFs. After treatment(s), MEFs were collected and isolated into cytoplasmic and nuclear fractions before being analyzed for sphinganine-1-phosphate (Sa1P) by LC-ESI-MS/MS. Data are shown as mean $\pm$ SEM (n=3 for each group). # The mean level of Sa1P in cytoplasmic or nuclear FB1-treated LM/Bc MEFs was significantly higher than the Sa1P level in the same fraction of control LM/Bc MEFs ($p < 0.04$). **The mean level of nuclear Sa1P in LM/Bc MEFs treated with ABC294640 and FB1 was significantly lower than the level found in the nucleus of FB1-treated MEFs ($p \leq 0.05$). ‡ The mean level of Sa1P in the cytoplasmic or nuclear fraction of LM/Bc MEFs treated with ABC294640 and FB1 was significantly higher than LM/Bc control MEFs ($p \leq 0.05$). † The mean level of Sa1P in the nuclear fraction of LM/Bc MEFs was significantly more than the mean level of Sa1P in the cytoplasmic fraction ($p \leq 0.05$) after FB1 treatment. ND signifies that the analyte was either not detected or present in an amount that could not be quantified (trace amount).

FIGURE 7:

FIGURE 7: Sa1P levels after treatment with PF-543, a selective Sphk1 inhibitor, and FB1 in LM/Bc MEFs. After treatment(s), MEFs were collected and isolated into cytoplasmic and nuclear fractions before being analyzed for sphinganine-1-phosphate (Sa1P) by LC-ESI-MS/MS. Data are shown as mean $\pm$ SEM (n=3 for each group). # The mean level of Sa1P in cytoplasmic or nuclear FB1-treated LM/Bc MEFs was significantly higher than the Sa1P level in the same fraction of control LM/Bc MEFs ($p \leq 0.005$). **The mean level of nuclear Sa1P in LM/Bc MEFs treated with PF-543 and FB1 was significantly lower than the level found in the nucleus of FB1 only-treated MEFs ($p \leq 0.02$). ‡ The mean level of Sa1P in the cytoplasmic or nuclear fraction of LM/Bc MEFs treated with PF-543 and FB1 was significantly higher than LM/Bc control MEFs ($p \leq 0.01$). † The mean level of Sa1P in the nuclear fraction of LM/Bc MEFs was significantly more than the mean level of Sa1P in the cytoplasmic fraction ($p \leq 0.002$). ND signifies that the analyte was either not detected or present in an amount that could not be quantified (trace amount).

FIGURE 8:

FIGURE 8: Sa1P levels after concurrent treatment with PF-543, ABC294640, and FB1 in LM/Bc MEFs. After treatment(s), MEFs were collected and isolated into cytoplasmic and nuclear fractions before being analyzed for sphinganine-1-phosphate (Sa1P) by LC-ESI-MS/MS. Data are shown as mean $\pm$ SEM (n=3 for each group). #The mean level of Sa1P in cytoplasmic or nuclear FB1-treated LM/Bc MEFs was significantly higher than the Sa1P level in the same fraction of control LM/Bc MEFs ($p \leq 0.005$). **The mean level of Sa1P in the cytoplasmic or nuclear fraction of LM/Bc MEFs treated with PF-543, ABC294640, and FB1 was significantly lower than the level found in the same fraction of FB1-treated MEFs ($p \leq 0.005$). ‡ The mean level of cytoplasmic Sa1P in LM/Bc MEFs treated with PF-543, ABC294640, and FB1 was significantly higher than the level in LM/Bc control MEFs ($p < 0.01$). †The mean level of Sa1P in the nuclear fraction of FB1-treated LM/Bc MEFs was significantly more than the level of Sa1P in the cytoplasmic fraction ($p = 0.0007$). However, in LM/Bc MEFs treated with both Sphk inhibitors and FB1, the mean level of cytoplasmic Sa1P was significantly more than nuclear Sa1P ($\diamond$, $p = 0.0094$). ND signifies that the analyte was either not detected or present in an amount that could not be quantified (trace amount).