Ceramide synthase inhibition by fumonisins: a perfect storm of perturbed sphingolipid metabolism, signaling and disease

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for *The Journal of Lipid Research* Perspectives, 2019

Supplement B. Additional information and references for Figure 3.

The following pages expand the legend for Figure 3 with brief explanations of each item and a bibliography that, although somewhat large, still does not claim to be all-inclusive. The purpose of the figure is to illustrate how inhibition of CerS causes changes in many types of sphingolipids, then to summarize some of the biological functions that have been associated with these sphingolipids, and lastly, to list the major disruptions in cell behavior that have been reported to be caused by fumonisins. There is considerable overlap, and some redundancy, of the information in the middle and right columns to illustrate how numerous similarities between sphingolipid regulation and fumonisin effects lead to the conclusion that CerS inhibition is the primary effect of fumonisins in causing disease. Some useful reviews of this topic are references (1-5).

No alternative targets of FB1 have been established, although a few have been suggested (PKC, for example) (6) and a computational analysis proposed that the N-acyl-metabolites could associate with other binding partners such as CERT and sphingosine kinase (7), but none of these have been experimentally verified as having a role in the biochemical disruption or pathophysiologic effects of FB1. Fumonisins undoubtedly interact with other cellular components, but it is unlikely that other targets, if found, will supplant the importance of CerS inhibition to disease.

Abbreviations: 1dSa, 1-deoxysphinganine; AP1, Fumonisin B1 aminopentol or hydrolyzed FB1; Cer, ceramide; CerS, ceramide synthase; DHCer, dihydroceramide; ER, endoplasmic reticulum; FB1, Fumonisin B1; GSL, glycosphingolipids; HFB1, hydrolyzed FB1; PKC, protein kinase C; S1P, sphingosine 1-phosphate; Sa, sphinganine; Sa1P, sphinganine 1-phosphate; SL, sphingolipid; SM, sphingomyelin; So, sphingosine; SPT, serine palmitoyltransferase. **Note: the abbreviations SphB and SphB1P have been used where there are multiple sphingoid bases and 1-phosphates that are likely to be encompassed by the abbreviation, not just Sa and So.**

Altered sphingolipid metabolites (left column)

Typically elevated:

Sphingoid bases (Sa, 1-deoxySa, So, etc.)—These are elevated as substrates of CerS that accumulate when CerS is inhibited by fumonisins.

Sphingoid base 1-P (Sa1P, S1P)—These are elevated as a consequence of the accumulation of substrates for sphingosine kinases.

Typically decreased:

Ceramides (dihydroCer, 1-deoxyDHCer)--These typically decrease upon CerS inhibition by fumonisins, although DHCer are usually affected first because cells contain large amounts of complex SL (in particular, SM) that initially "buffers" changes in Cer.

SM & Glycosphingolipids—These all decrease eventually upon CerS inhibition by fumonisins, but the magnitude depends on the amounts that the cells contain, the rate of biosynthesis versus turnover, and the amounts that are taken up from outside the cell (and used intact or recycled) versus made *de novo*. One noteworthy exception is a report that in Swiss 3T3 cells, long-term treatment with FB1 up-regulated neutral glycosphingolipid synthesis (8).

References for these changes have been given in the main text and the following sections. A question that is often pondered is: Are the effects of fumonisins mainly due to elevation of one particular subcategory of metabolite--Sa and Sa1P, perhaps, because they typically display the biggest changes? Or, are elevations in all SphB (Sa, So and 1dSa) and SphB1P (Sa1P, S1P)[1](9) important? How important is the elevation of 1dSa? How significant is the reduction of Cer and/or complex SL/GSL? It is nearly impossible to distinguish these possibilities because in altering one, many of the others change, too (perturbing one enzyme of sphingolipid metabolism often initiates a "whack-a-mole" game of other bioactive sphingolipids popping up).

[1] This is not meant to imply that the effects of all of these compounds will be the same (e.g., Sa1P and S1P, etc.). Indeed, there are numerous cases in which they are different, such as in studies of human embryonic stem cell-derived neural epithelial progenitor (hES-NEP) cells (reference #9), where Sa1P was a more potent stimulator of inhibition of cAMP and Smad phosphorylation than was S1P.

Intermediate biochemical responses (middle column)

Sphingolipid signaling targets:

For additional background information about SL metabolism, signaling and disease—and for useful summarizing diagrams and tables that help integrate these processes—see reference (10).

Protein kinases

Protein kinase C: The protein kinase C family enzymes participate in a wide range of physiologic processes, many with relevance to cell growth regulation, differentiation and cancer (11). Sa, So and most other SphB inhibit the "typical" protein kinase C isoenzymes (i.e., those activated by diacylglycerol and some other lipids) (12-14) and FB1 has been shown to reduce PKC activity both by elevation of endogenous SphB (15) and—over longer term--by altering PKC expression (16).

Atypical protein kinase C: The atypical PKCs (aPKC, which include PKCζ and PKCι/λ) participate in cell motility, survival regulation (including PKB/Akt and suppression of apoptosis) and a wide-range of other processes (11, 17). Cer activates PKCζ (zeta) (18) and mediates the interaction between PKCζ and its inhibitor protein, PAR-4; therefore, it can serve to activate or inhibit kinase activity depending on whether PAR-4 is present (19). *De novo* Cer biosynthesis has also been reported to be required for activation of aPKC in neural progenitor cell motility and brain development (20).

Cer and aPKC are important regulators of primary cilia formation and signaling (21). The primary cilium (in contrast to motile cilia) is present on most cells in usually one copy per cell and serves as a sensory organelle (22) for mechanical forces and chemical stimuli for a wide range of functions (for example, in brain these include early patterning in neurogenesis, the proliferation and differentiation of neural stem/progenitors in fetuses and adults, and the maturation of neurons and tumorigenesis). Cer is critical for the regulation of primary cilia formation and elongation via aPKC (21) and to inhibit TGF-β receptor I/II trafficking and signaling at primary cilia to attenuate cell migration and tumor metastasis (23). In studies of Madin-Darby canine

kidney cells (MCDK) (21), inhibition of CerS by FB1 prevented co-distribution of aPKC and Cdc42 in the centrosomal/pericentriolar region of the cells and severely impaired ciliogenesis.

FB1 has been reported to block many processes that elevate Cer to activate PKCζ (24), which could participate in some aspects of the cytotoxicity of 1-dSa (25). Interestingly, however, S1P and Sa1P have been recently discovered to activate aPKC (26), which could provide another perspective on how aPKC participate in some of the cellular effects of fumonisins.

Fam20c (also called DMP-4, dentin matrix protein 4, and G-CK, Golgi casein kinase): So (and Sa) activate Fam20c, a luminal Golgi protein kinase that is thought to be responsible for putting the phosphate groups on most secreted proteins, such as PCSK9, ApoA2, ApoB, ApoE, ApoL1, and ApoA5 in humans (27).

Mitogen-activated protein kinases (MAPK): Increased MAPK activity was seen in Swiss 3T3 cells after addition of FB1 (75 μM), with the first detectable change ~4 min, the highest elevation between 10-15 min and a substantial drop by 30 min (28). This is a timeframe similar to MAPK (ERK) activation by So (29). Thus, MAPK(ERK) activation might involve SphB elevation by FB1, if it occurs rapidly enough under the conditions of this study. Indeed, in a study using monkey kidney MARC-145 cells as a model for fumonisin-induced nephrotoxicity, FB1 was found to activate ERN1-MAPK8/9/10 via disruption of SL biosynthesis because it was blocked by myriocin (30). Structural studies of MAP kinase p38α, which is activated in many mammalian cells in response to cellular stress from inflammatory cytokines, ischemia and DNA damage, suggest that it's hydrophobic binding pocket might accommodate long-chain aliphatic compounds such as Cer (31).

Protein kinase A type II: Sa and So (but not S1P nor Cer) activate PKA type II via a mechanism that is independent of cAMP (32) and does not involve the dissociation of PKA holoenzyme into catalytic and regulatory subunits (as does cAMP) which accounts for basal membrane-associated PKA activity. One of the targets of Sa and So activated PKA type II is the multifunctional adapter protein 14-3-3ζ, which is very interesting because the 14-3-3 proteins have been described as being "at the center of

the signaling hub that governs critical processes in cancer, including apoptosis, cell cycle progression, autophagy, glucose metabolism, and cell motility" (33).

Another provocative connection between the SL pathways and PKA is that PKA type I interacts with SKIP (SPHKAP), sphingosine kinase-interacting protein (34), which anchors the type I PKA holoenzyme at mitochondria where it can phosphorylate the inner mitochondrial membrane protein ChChd3 (Coil-Coil Domain Protein 3) (35), an inner mitochondrial membrane protein that is essential for maintaining crista integrity and mitochondrial function (36). SKIP was initially identified as a negative regulator of sphingosine kinase 1 (as well as a new AKAP, protein kinase A anchor protein) (37); therefore, one can envision conditions where interference with sphingosine kinase activity by SKIP might influence the fate of elevated SphB in cells exposed to fumonisins, and/or the corrollary, that elevation of SphK by fumonisins (38) alters the availability of SKIP to interact with ChChd3.

Phosphoprotein phosphatases (PP)

PP1: Cer is an activator of PP1 (10) and some of the phosphoprotein substrates and signaling events that have been associated with this activation are ezrin and cell migration, the dephosphorylation of SR proteins in response to FAS activation (39); b) alternative splicing of caspase 9 and Bcl-x (40); and c) translocation of PKCα/βII to the pericentrion after sustained activation (41). FB1 inhibited all of these effects (39-41).

PP2A: Cer is an activator of PP2A (10) and some of the proteins that have been identified as substrates are protein kinase C (PKC), protein kinase B (AKT), and ezrin. Inhibition of AMPK by ethanol has been demonstrated to be attenuated by FB1 (42). A transcriptomic and kinomic analysis of samples from liver and intestine from pigs fed a fumonisin contaminated diet concluded that most of the effects of FB1 are mediated by the regulation of Cer metabolism and its influence on PP2A and the phosphoinositide 3-kinase (PI3K)/AKT signaling pathway (43). Cer regulates the interaction of PP2A with its inhibitor SET (I2PP2A), which could link it to regulatory T cell functions (10).

Other PP: A study of the effects of FB1 on the *in vitro* phosphoprotein phosphatase activity of PP1, PP2A, PP2B, PP2C and PP5/T/K/H found inhibition of all five with IC50 values of 80 to 3000 μM (PP5 was most sensitive) (44). This effect

appears to be independent of CerS inhibition, but has not been confirmed by others. It would be interesting to examine this again, especially with N-acyl-fumonisins.

S1P receptors & pathways

SphB1P are involved in a wide range of cell regulatory processes through both signaling via cell surface receptors (S1PR) and intracellular signaling pathways (45, 46).

S1P receptors: Five cell surface G-protein-coupled S1PRs mediate a wide range of biological processes (gene expression, autophagy, endocytosis, and others) and function in cell growth and survival, adhesion, migration and invasion, angiogenesis and lymphangiogenesis, endothelial and epithelial barrier function, lymphocyte function and trafficking, and multiple aspects of cancer. The roles that are played depend on both the S1P concentration and the specific S1PR on the cells, with two examples (from the review (47)) being: a) maintenance of endothelial barrier integrity, which has been linked to S1P that is in circulation as an ApoM–S1P–HDL complex that is secreted by liver and activates S1PR1 on endothelial cells; and b) liver fibrosis, where there is evidence for many factors being possible contributors, such as S1P transport from hepatocytes to signal through S1PR2 to promote fibrosis in an autocrine loop, S1P recruitment of hepatic stellate cells via S1PR3, as well as other mechanisms. In brain, S1P also has multiple functions and has been described as a “double-edged sword” (48) because it is involved in synaptic transmission, neuronal autophagy and neuroinflammation, plus it is an activator of BACE1 (β-site APP cleaving enzyme-1), the rate-limiting enzyme for amyloid-β peptide (Aβ) production (this binding appears to be in the BACE1 transmembrane/intracellular domain) (49).

S1P has been recently reported to activate of transcription factor SNAI2 via S1PR2 and S1PR3 receptors on MCF7 breast cancer cells (50). SNAI2 is a member of the Snail superfamily of C2H2-type zinc finger transcription factors that is one of the transcriptional regulators of the epithelial to mesenchymal transition (EMT) that has important roles in development and cancer. Therefore, the effect of S1P activation of SNAI2 is to increase cell invasion and metastasis. Interestingly, Cer (specifically C16-Cer) was reported (51) at almost the same time to down regulate SNAI2 expression. These studies had considered C16-Cer because reduced expression of CerS6 (which

makes C16-Cer) has been associated with apoptosis resistance and CerS6 had also been implicated in EMT. They (51) found that SNAI2 expression increased when there was shRNA-mediated knockdown of CerS 6 (or CerS5, the other CerS that makes C16-Cer) but there was no suppression by reduction of other CerS; furthermore, only C16-Cer restored suppression of SNAI2 (51). Therefore, SNAI2 appears to be under the type of control that has been referred to as a "sphingolipid rheostat" (52) wherein one metabolite activates a process (S1P activation of SNAI1) and another (Cer) having the opposite effect. In the earlier example of the rheostat, the processes were induction of apoptosis by Cer and inhibition of apoptosis by S1P.

As these discussions illustrate, these metabolites are involved in complex regulatory mechanisms and the disruption of SL metabolism by fumonisins to elevate S1P and Sa1P while reducing Cer appears to be ideal for disruption of the important pathways that they regulate.

Intracellular proteins that interact with S1P: Less is known about the intracellular proteins that interact directly with SphB1P, but the ones that have been found so far—which include BACE1, mentioned above, histone deacetylases (HDACs) (53), TRAF2 (a key component in NF-κB signaling) (54) and atypical protein kinase Cs (26)—have important functions in gene regulation, cell motility, survival regulation, immune regulation, and other processes that could contribute to the disruption of cell behavior when SphB1P are elevated by fumonisins.

Note that the list of cell types and disorders for which S1P regulation is known to be very important overlaps with organs adversely affected by fumonisins--endothelial cells, brain, liver, and cancer (kidney disease would be also be included (55)). S1P also has important functions in embryonic development because SphK1/SphK2 double-knockout mice (56) produce embryos displaying vascular defects and exencephaly, a cranial neural tube defect (NTD). According to the authors of this study, the development of NTD in the SphK1/SphK2 embryos appeared to be due to an imbalance between proliferation and cell survival.

Other signaling pathways

Programmed cell death mechanisms: SL play multiple roles in the regulation of apoptosis, necroptosis, autophagic cell death, and others stress response mechanisms that result in programmed cell death. Cer is prominent in many of these via destabilization of mitochondrial membranes (as discussed below), activation of pro-apoptotic signaling mechanisms (e.g., Bax and Caspases), induction of autophagy, etc. as has been reviewed in depth (10) and specifically in relation to cancer (57).

Prominent among the Cer signaling pathways has been the activation of PP1 and PP2A (two targets of Cer discussed above) that have also been referred to as "ceramide-activated protein phosphatases" (CAPP). An example of a downstream process that is turned on by CAPP is the alternative 5′splice site selection of Bcl-x pre-mRNA to produce either pro-apoptotic Bcl-x(s) or anti-apoptotic Bcl-x(L). Generation of Cer by *de novo* biosynthesis has been shown to decrease Bcl-x(L) mRNA with a concomitant increase in Bcl-x(s) mRNA in A549 cells (58), and thus altering cell fate. Another example of alternative splicing that is related to Cer via CAPP activation concerns p53 ("guardian of the genome"). This important tumor suppressor is often lost or defective in cancer, but this is sometimes due to defective pre-mRNA splicing, and Cer activation of PP1 to restore splicing to wild-type p53 and induce p53-dependent apoptosis (59). Fumonisin-induced loss of these functions could increase cancer risk by allowing damaged cells to survive that otherwise might be eliminated before becoming cancerous.

These examples are only a fraction of the known mechanisms linking SL with programmed cell death pathways. For a broader discussion of this topic, see references (10) and (57).

Cathepsins and non-Caspase-dependent apoptosis: Cathepsins are lysosomal proteases that are typically made as pro-peptides that are cleaved to unmask the fully active catalytic domain. Cer binds directly to cathepsin D to induce autocatalytic proteolysis of pre-pro cathepsin D to form the enzymatically active 48/32 kDa isoforms (60). Both cathepsin B and cathepsin D appear to participate in the Cer-mediated cell death induced by the lysosomotropic acid ceramidase inhibitor LCL521

(61). Lysosomal Cer triggers cytosolic cathepsin B-mediated degradation of an inhibitor of apoptosis protein in inducing apoptosis in natural killer/T lymphoma cells (62). SphB have also been reported to activate cathepsins (63) and to play a role in sphingosine-induced apoptosis (64). Many of these effects are mediated by permeabilization of lysosomes, which releases these and other degradative enzymes into the cytosol (65).

"Signaling" by free sphingoid bases: Although the finding that So inhibits PKC (12) is considered the "classic" discovery that set the sphingolipid signaling field in motion (66), cell regulation by free So and other SphB has been the most elusive to pin down. In large part, this has been due to the rapid conversion of SphB to the N-acyl-derivatives and 1-phosphates. Their targets range from PKC (as described above) to ion transporters (such as triggering release of Ca^{2+} from lysosomes) (67) and their cellular effects span inhibition of growth, enhancement of differentiation and induction of apoptosis (10). Studies with poorly metabolized SphB (such as the L-*threo*-stereoisomer) (14, 68, 69) and other analogs (70) have established that SphB *per se* are growth inhibitory and cytotoxic for cancer cells, so the elevation of these compounds by fumonisins is expected to have deleterious effects. At very high concentrations, SphB can be cytotoxic by virtue of their biophysical properties in membranes (71) which can result in their having lysosomotropic effects (72) that—among other things—can release lysosomal proteases (e.g., cathepsins) into the cytosol and elevate production of reactive oxygen species (73).

One of the additionally puzzling findings with mice (74) and humans (75) that were administered Safingol (L-*threo*-sphinganine) is that it was N-methylated, which raises the possibility that N-methyl-SphB might be additional side-products of interest in animals exposed to fumonisins.

"Crosstalk "with other signaling pathways: There are numerous instances where there is "cross-talk" between SL and other cell signaling pathways. For examples:

cAMP & PKA. Sphingosine has been reported to have multiple non-specific effects on adenylate cyclase and cyclic AMP accumulation (76), and S1P induces the translocation of PAM (Protein Associated with Myc) from the ER to the plasma membrane where it inhibits adenylyl cyclase activity. PAM is localized at the

endoplasmic reticulum in HeLa cells and is recruited to the plasma membrane upon serum treatment, causing an inhibition of adenylyl cyclase activity (77). Also noted above, SphB activate PKA type II via a mechanism that is independent of cAMP (32).

PLA2 and arachidonic acid metabolism. There are multiple mechanisms whereby SL affect the arachidonic acid (AA) cascade (78). Cer is a mediator of tumor necrosis factor (TNF) stimulation cPLA2 and, when added exogenously, increases expression of cPLA2 and cyclooxygenase-2 (COX-2) mRNAs (79). Other connections (78) include activation of $cPLA_2\alpha$ by Cer1P and lactosylceramide versus inhibition by SM. The Cer1P receptor is coupled to G_i proteins and activates of the PI3K/Akt and MEK/ERK1-2 pathways, therefore, might provide a link with cell proliferation and survival.

S1P induces COX-2 expression, and was noted to account for the antiproliferative properties of S1P in human hepatic myofibroblasts which are cells that are important in the development of fibrosis during liver injury (80). Therefore, two bioactive sphingolipids have been reported to coordinate production of prostaglandin E2 (PGE_2)--Cer1P for activation of PLA2 followed by S1P for COX-2 (81). Interestingly, Sa1P has been reported not to induce (COX-2), but rather to activate ERK1/2 and up-regulate expression of matrix metalloproteinase 1 (MMP1) in response to TNFα (82).

Comment: Fumonisins have been reported to induce down-regulation of cytoplasmic cPLA2 activity and AA metabolism by a mechanism involving prostaglandin production, cAMP synthesis and PKA (83). These observations might also be related to the effects of SphB, Cer and the 1-phosphates described above.

Cer1P signaling. Although Cer1P amounts have not been reported in any fumonisin studies (as far as we are aware), it is another bioactive metabolite that displays crosstalk with other lipid signaling pathways (84, 85). For example, Cer1P has been found to stimulate the release of vascular endothelial growth factor (VEGF) by macrophages via activation of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B and MAPK-kinase (MEK)/extracellularly regulated kinase-2 (ERK-2) pathways (86).

Diacylglycerol metabolism and SM biosynthesis. As noted below under lipid pathways affected by alteration of sphingolipid metabolism, one direct connection between a signaling sphingolipid (Cer) and a signaling glycerolipid (diacylglycerols, DAG, which are activators of PKC) is the substrate/product equation of the SM synthases (Cer + PC ← → SM + DAG) (87). SM synthase has also been noted to affect lipid droplet formation (88), which is being associated with many disease states.

CERT-related steroidogenic acute regulatory protein D7 (StarD7). This is a Cer-binding protein that was found using a Cer analog that can be cross-linked to proteins via both photoactivatable and clickable functional groups (89). StarD7 is required for efficient mitochondrial import of phosphatidylcholine and plays a role in mitochondrial function and morphology.

This list is not all-inclusive, but provides an illustration of how sphingolipids are widely interconnected with other cell signaling pathways. For some additional examples, see reference (10).

Nuclear receptor regulation and epigenetic controls

Nuclear receptors: Nuclear receptors are ligand-activated transcription factors that have been found to bind a wide range of lipids, including cholesterol, phospholipids, SL, and other amphipathic molecules that can functionally regulate their activity (90). The SF1 nuclear receptor—a major regulator of glucocorticoid production--appears to be regulated by So and S1P as well as other nuclear lipids (90-92). The converse is also known—i.e., for nuclear receptors to utilize SL metabolism and signaling as part of their mechanism of action, which has been observed in the induction of ABCA1-mediated cholesterol efflux by the LXR agonist 22(R)-hydroxycholesterol in macrophages, which requires activation of So kinase and S1P production (93). FB1 has been noted to have more pronounced hepatotoxicity for $LXR^{-/-}$ mice compared to $LXR^{+/+}$ mice (94) and this was suggested to be "an alternative mechanism not related to the inhibition of sphingolipid synthesis for mycotoxin toxicity"; however, if LXR is involved in regulation of sphingosine kinases (as suggested by the findings with

macrophages), then the LXR$^{-/-}$ mice might have elevated cytotoxic SphB versus their phosphorylated metabolites.

Another example of an affected nuclear receptor is the androgen receptor, for which non-cytotoxic concentrations of FB1 had an antagonistic effect on the androgen nuclear receptor transcriptional activity at 10 μM, and no agonist responses were seen at this concentration (95).

Histone deacetylase: Acetylation of histones on lysine residues by histone acetyltransferases (HATs) and removal of the acetyl groups by histone deacetylases (HDACs) are major determinants (activating vs inhibiting, respectively) of transcriptionally active chromatin domains. S1P and Sa1P have been shown (53) to inhibit the activity of histone deacetylases HDAC1 and HDAC2 (but have no effect on HAT activity), and thereby prevent the removal of acetyl groups from lysine residues that would suppress transcription. Nuclear S1P is produced predominantly by SphK2 (96), which was found (53) to associate with HDAC1 and HDAC2 in repressor complexes that are selectively enriched at the promoters of the genes encoding the cyclin-dependent kinase inhibitor p21 or the transcriptional regulator c-fos. Therefore, elevation of nuclear SphB1P will enhanced local histone H3 acetylation and transcription (53). Nuclear S1P from Sphk2 also inhibits DNA synthesis and cell growth (96, 97).

FB1 has been found to elevate nuclear nuclear Sa1P and decrease HDAC activity in mouse embryonic fibroblasts (98).

Rb dephosphorylation: Rb dephosphorylation results in growth inhibition. So has been shown to induce Rb dephosphorylation in a stereoselective manner (68). In these studies, the effect of So did not appear to be due to its conversion to Cer; however, Cer has also been shown (99) to induce Rb hypophosphorylation leading to growth arrest and cellular senescence. In this case, Cer inhibited cyclin-dependent kinase CDK2 activity but via activation of p21, a CDK-inhibitory protein.

FB1 has been shown to repress CDK2 activity in monkey kidney cells (CV-1) and to induce p21(Waf1/Cip1) as well as other CDK inhibitors, p27(Kip1) and p57(Kip2) (100).

p53: The p53 protein (coded for by the TP53 gene) is an important tumor suppressor that is often referred to as “the guardian of the genome” because it functions as a transcription activator to mediate cellular responses to DNA damage and other stresses that could result in genomic damage. Cer (particularly C16-Cer) activates p53 by binding within the p53 DNA-binding domain (101), which disrupts its complex with E3 ligase MDM2 that would otherwise cause p53 ubiquitination and proteasomal degradation. Interestingly, C16-Cer biosynthesis *de novo* (102) is induced as part of the non-genotoxic p53-dependent cellular stress response (and transcriptional up-regulation of CerS6 is dependent on p53) (103). One of the inducers is folate deficiency (101, 102), with activation of p53 resulting in apoptosis (104). It has been proposed that stabilization of p53 by C16-Cer binding is utilized for cellular response to nutrient/metabolic stress (105). Pretreatment with FB1 to block C16-Cer accumulation rescued cancer cells in culture from apoptosis (104), but it is not clear if this plays a role in FB1 toxicity and carcinogenicity *in vivo* (see (106) and discussion below).

Membrane functions influenced by sphingolipids:

Membrane structure

Membrane fluidity and transient nanodomains (“rafts”): Biological membranes contain mainly complex SL—sphingomyelins (SM)(107-109), and neutral and ionic glycosphingolipids (GSL) which are comprised of hundreds of subspecies including mono-, di- and poly-hexosyl-Cer (110, 111) , sulfatides (112, 113) and gangliosides (114, 115). Each molecular species contributes special features to membrane bilayers, affecting the fluidity, thickness, curvature, surface charge, ability to be recognized by “lectins” (a general term for carbohydrate binding proteins, but in mammals encompasses membrane receptors, transporters, extracellular matrix proteins, circulating proteins, etc.). The simpler SL (SphB, Cer, etc.) can also affect membrane structure if present in sufficient amounts (71, 109). An important function of sphingolipids is to form “rafts”(116), a controversial term applied to the tendency of SL to associate with each other and some additional lipids (e.g., cholesterol) to cluster with proteins in the otherwise “fluid” membrane (these have interchangeably been called “rafts,” “microdomains,” “ultrananodomains” (117) and more recently, "transient

nanodomains" (118)). These are thought to be important because it affects the properties of receptors and transporters associated with these regions of the membrane. The SL backbones (Cer, SphB) also have significant effects on the properties of membranes (119), and the 1-deoxySL (e.g., 1dDHCer) are highly disruptive of membrane structure (120).

Lysosomotrophic effects: SphB tend to be membrane disruptive when added at high concentration because they have a relatively high critical micelle concentration (~1 μM) and can serve as a single chain amphiphile at high concentrations (i.e., behave like a detergent). They are also "trapped" in acidic compartments and can have lysosomotropic effects (72, 121), rupturing lysosomes at high concentrations.

Membrane-associated regulatory processes

Plasma membrane receptors and transporters: Sphingolipids are important to plasma membrane structure in general, and particularly via their formation of transient nanodomains ("rafts") that are thought to be important for the function of many receptors and membrane transporters (116). Thus, one would predict that alteration of the SL composition by fumonisins would perturb the function of some of these proteins. Some examples of where this has been seen are:

a) the serotonin1A receptor: this G-protein coupled receptor is implicated in the generation and modulation of various cognitive, behavioral and developmental functions, and was shown to be impaired upon depletion of SL using FB1 (122, 123);

b) the co-receptor function of ganglioside GM1 for fibroblast growth factor 2 (FGF2) and its cell surface receptor, where reduction of GM1 by FB1 prevented the interaction of FGF2 with cells and its biological activity as a growth factor (124);

c) the endocannabinoid receptor signaling: FB1 (with L-cycloserine, an SPT inhibitor) reduced the effects of anandamide (one of the endocannabinoids that affects cell behavior by binding to CB1 and CB2 receptors) on cell viability and p38 MAPK phosphorylation (125); and

d) the folate receptor, which is normally associated with "rafts" (like many glycosylphosphatidylinositol-anchored proteins), and lost almost all folate receptor-mediated transport of 5-methyltetrahydrofolate when cellular SL were reduced by

approximately 40% by FB1 (126). FB1 was also found to inhibits integrin-mediated cell-matrix adhesion of B16-BL6 cells to immobilized fibronectin (127).

Another likely receptor to be affected by fumonisin is the epidermal growth factor (EGF) receptor since its activity is modulated by ganglioside GM3 (128-130). Swiss 3T3 fibroblasts treated with FB1 underwent visible changes in morphology that were reversed by adding back GM3 (131), and these seemed to be directly connected to functions of GM3 for the cells. In contrast, the disruption of DNA synthesis and cell proliferation was not reversed by GM3, so those effects of FB1 are probably due to other metabolites. In other studies, the effects of FB1 have been seen to depend on the growth factor and receptor type because for Hepa1-6 cells GM3 inhibited EGF-stimulated motility, but promoted hepatocyte growth factor receptor (HGFR/cMet)-stimulated motility (132).

ER regulatory mechanisms sensitive to SL: These range from processes that are involved in "normal" functions (e.g., regulation of lipid metabolism) to stress mechanisms that can trigger cell death.

ORMDL proteins: SPT activity is regulated by inhibitory Orm/ORMDL proteins (133-135). Cer appears to be a regulator of Orm/ORMDL, so depletion of Cer by fumonisins probably sustains, and even increases, SPT activity.

TM4SF20: Cer regulates (Transmembrane 4 L six Family Member 20) by inverting the topology of the protein from having the N-terminal transmembrane helix in ER lumen--the topology termed TM4SF20(A)--to having the N-terminal transmembrane helix located on the cytosolic side of the ER--the topology termed TM4SF20(B) (136, 137). This translocation sometimes appears to require a protein with a Cer-binding domain (TRAM2 (translocating chain-associated membrane protein 2). TM4SF20(A) inhibits the proteolytic activation of CREB3L1 (cAMP Responsive Element Binding Protein 3-Like 1), a membrane-bound transcription factor; whereas, TM4SF20(B) stimulates proteolytic activation of CREB3L1, allowing it to drive expression of genes that inhibit cell proliferation and turn on assembly of collagen-containing matrix.

ER Stress: SL of multiple classes can cause ER stress when metabolism is disrupted, and they also serve as mediators of the ER stress mechanisms in diseases such as diabetes. As two examples: DHCer delay cell cycle G1/S transition via

activation of ER stress and induction of autophagy (138); and Cer induce lipotoxicity and hypothalamic ER stress, leading to sympathetic inhibition, reduced brown adipose tissue (BAT) thermogenesis, and weight gain (139). The mechanisms of sphingolipid-mediated ER stress appear to be worked out in the greatest detail in yeast (140).

Vesicular trafficking: Cer has been known for several decades to be concentrated in Golgi as well as to influence ER/Golgi trafficking (141), and more recent studies have uncovered requirements also for SM (at the TGN) (142, 143), S1P for endocytosis and trafficking (144) and multiple sphingolipids in the biogenesis and biological activity of extracellular vesicles (including exosomes) (145, 146). Sphingolipids are also key players in determining if vesicles associate with autophagosomes (147).

FB1 probably affects many of these processes. At the level of the partitioning of the *de novo* biosynthesized metabolites, FB1 was noticed to cause greater inhibition of SM biosynthesis than glycosphingolipid biosynthesis (148). This might reflect depletion of *de novo* biosynthesized Cer before it reaches the sites of SM biosynthesis because the enzymes for biosynthesis of Cer and galactosylCer are located in the ER (the latter in the ER lumen) and glucosylCer in the nearby *cis*-Golgi; whereas, SM biosynthesis occurs primarily in the *trans*-Golgi and plasma membrane, to which Cer is transported via the transport protein CERT and vesicular trafficking (149).

FB1 has also been found to inhibit the export of the nicotinic acetylcholine receptor (AChR, a prototype ligand-gated ion channel), which was retained in the ER (150), and the transport of a lysosomal protein (prosaposin) to lysosomes (151). Somewhat surprisingly, FB1 does not appear to affect exosome release nor content (146).

Mitochondrial membrane permeability (and related aspects of mitochondrial sphingolipidology): Mitochondria have many important functions in cells that overlap with sphingolipid metabolism and function, including the housing of important anabolic and catabolic pathways (e.g., connected via palmitate and serine metabolism), the conversion of energy from multiple sources into ATP (sphingolipids can affect the mitochondrial electron transport pathway, as discussed later in this Supplement), and regulation of key steps of the intrinsic apoptosis pathway (discussed here). In the latter

capacity, Cer participate in the mechanism(s) of mitochondrial outer membrane permeabilization (MOMP) that produce pores that are sufficiently large to release proteins such as cytochrome c from the intermembrane space (i.e., between the inner and outer mitochondrial membranes) into the cytosol where they activate the apoptotic cell death machinery (caspases, endonucleases, etc.). Cer have been clearly shown to participate in MOMP and two mechanisms have been proposed for its action--generation of Cer channels (first proposed in reference (152) and recently reviewed (153)) and/or by interaction with a more complicated complex of regulatory proteins in the Bax/Bcl-2 category with Cer and other membrane components, which have been described in several excellent reviews (154-156). The subject remains highly controversial (157), but for the purpose of this review the central point is uncontested—i.e., that changes in mitochondrial Cer participate in the induction of apoptosis. Cer has also been reported to induce lethal mitophagy (158) (and autophagy, see next section).

Indeed, many sphingolipids have been associated with mitochondrial apoptosis, from SphB to gangliosides. So induced apoptosis (159, 160) does not appear to be due to its conversion to Cer because FB1 did not block the effect (as reviewed in (160)). Mitochondrial S1P produced from SphK2 also promotes apoptosis (161). The apoptosis attributed to gangliosides involves glycosphingolipid-enriched microdomains in mitochondrial associated membranes (MAM) that link ER stress to Ca^{2+}-dependent mitochondrial apoptosis (162).

Studies of the effects of FB1 on mitochondrial and MAM CerS found that the activity with Sa was inhibited but not activity with So (163). This is puzzling but perhaps reflective of the Sa being made in the *de novo* pathway which is accessible to FB1 versus So uptake from turnover.

Autophagy/mitophagy: Autophagy is a highly regulated process for degradation of cellular components as needed to provide essential amino acids and other nutrients, to dispose of unnecessary or dysfunctional components, such as damaged mitochondria (in mitophagy), and to assist the destruction of bacteria and other phagocytosed materials. Multiple SL (Cer, DHCer, S1P and probably others) are involved in the different subcategories of autophagy, as discussed in several well-written reviews on SL and autophagy (147, 164, 165) and mitophagy (166, 167). In

addition, in at least some cases, the induction of autophagy requires *de novo* Cer biosynthesis (168). Mitochondrial Cer has been demonstrated to directly interact with LC3BII and target autophagosomes to mitochondria to induce lethal mitophagy (158).

Both (DH)Cer and SphB1P are known to induce autophagy, so it is unclear how FB1 will affect this process; however, several studies (discussed below) have reported increased autophagy after FB1 administration (23)(169, 170).

Other lipids influenced by sphingolipids:

Cholesterol and other sterols

A "special" relationship between sphingolipids cholesterol has been known for decades, and occur on both the structural level where cholesterol interact with sphingomyelins, Cer, complex GSL (see ref. (107, 171) for review) in "transient nanodomain" (118) (also called "rafts"), and metabolism and transport. Examples of the latter are that Cer has been shown to increase the plasma membrane presence of ABCA1 and stimulate cholesterol efflux to ApoA-I (172); and S1P (added exogenously or made endogenously by Sphk stimulation by 22(R)-hydroxycholesterol) also enhanced cholesterol efflux via a signaling pathway that appeared to utilize the S1P3 receptor (93). Therefore, it is not surprising that fumonisin perturbation of the sphingolipid status of cells affects cholesterol amounts and location (173) (including raft composition) (174).

Phospholipids and glycerolipids

Phospholipids and glycerolipids (i.e., diacylglycerols and triacylglycerols) are also altered after fumonisin exposure and in most instances the effects appear to be related to the perturbation of SL metabolism. For example, analyses of J774 cells (175), HeLa cells (176) and rats (174) treated with FB1 found elevated phosphatidylethanolamines (PE) and the most comprehensive "lipidomic' study (176) noted that 1-alkylPE's were elevated. These are likely due to lytic cleavage of SphB1P, which produces ethanolamine-phosphate and fatty aldehydes that are reduced to fatty alcohols and incorporated into PE and 1-alkyl and alkenyl lipids, respectively (177). There are also

changes in some subspecies of phosphatidylcholines (PC), which might be expected due to the relationship between PC and SM biosynthesis (PC + Cer ←→ DAG + SM) (111) and, possibly, activation of phospholipase D by SL (178).

Fatty acids, aldehydes & alcohols

As noted in the text and above, lytic cleavage of SphB1P produces fatty aldehydes that are reduced to fatty alcohols and incorporated into 1-alkyl and alkenyl PE (177).

Other shifts in fatty acyl composition (chain length and desaturation) might be related to fumonisin's effects on the activity of ELOVL1 (179) (which interacts with CerS2) and the activity of Δ6-fatty acid desaturase (FADS2 or D6D) (174, 180). A direct link between CerS inhibition and changes in FADS2 activity has not yet been proven, but appears likely because FADS2 activity is affected by the ER membrane environment and ER stress (181), which are both affected by fumonisin. The unsaturated fatty acid status of liver influences hepatocarcinogenesis (173), therefore, CerS inhibition might be involved at many levels: perturbation of membrane structure, generation of bioactive metabolites, activiation key sphingolipid mediated-signaling pathways and cross-talk with other signaling pathways (e.g., PLA2 and COX-2).

Increases in lipid peroxidation is also common after fumonisin exposure (182) and is undoubtedly a consequence of production of reactive oxygen species (ROS) upon disruption of mitochondrial function by SL, as discussed elsewhere in this supplement.

Perspectives on proteins that interact with sphingolipids

New techniques have been developed over the past few years for trapping and imaging of binding partners for sphingolipids of all categories, from sphingoid bases (183) to Cer (184) and complex SL (185). It is expected that application of these technologies, and the generations that follow them, will further expand the number of proteins that bind these compounds and raise new questions about how they (and fumonisin) affect cell function.

Toxicologically relevant perturbations (right column)

As mentioned briefly in the main text, fumonisin is a proven cause of several farm animal diseases and is nephrotoxic and nephrocarcinogenic in rats and hepatoxic and hepatocarcinogenic in both rats and mice. Several authoritative bodies have evaluated or conducted safety assessments of fumonisin and these include the International Agency for Research on Cancer (186), the National Toxicology Program (187), the European Food Safety Authority (4) and, on three separate occasions, the FAO/WHO Joint Expert Committee on Food Additives (most recently as reference (5)). There have been many studies of the effects of fumonisins on cells in culture, organ slices (to more closely resemble cells *in vivo*), and tissues of animals exposed to specific fumonisins or culture materials with administration via multiple routes and protocols. The effects vary considerably for different cell types, organs, animal species, sex, strain and other factors. Nonetheless, in all *in vitro* and *in vivo* studies to date, where they have been analyzed, SphB and more recently, SphB1P have been found to be elevated and more complex SL decreased in a dose-dependent manner (for example, see references (187), (188) and (189)). This review has compiled the major biochemical and toxicologic effects so they can be contemplated from a mechanistic perspective. Other reviews with a mechanistic perspective include (3, 5, 190).

Programmed cell death pathways

Apoptosis: Numerous studies have reported that fumonisins cause cell death via apoptosis, a programmed cell death pathway for mammals. These include FB1 treatment of: primary (and neonatal) human keratinocytes, HET-1A human esophageal epithelial cells and HepG2 human hepatocarcinoma cells in culture (191, 192) and rat tubular epithelial cells after administration of FB1 in the diet (192); African green monkey kidney cells (CV-1) (193); pig kidney epithelial cells (LLC-PK1) (194); the human colonic cell line HT-29 (195); liver and kidney cells of Fischer rats and B6C3F1 mice (196); and human fibroblasts (197), among others (3, 5). In some studies, CerS inhibition/SphB elevation was established to play a role by inhibition of serine palmitoyltransferase (194, 195, 198).

The ability of FB1 to induce apoptosis and the protective effect of serine palmitoyltransferase inhibition on both SphB accumulation and apoptosis is used as support for a non-genotoxic mode of FB1 carcinogenicity that involves CerS-inhibition-dependent apoptotic necrosis, atrophy and consequent regeneration in rodent liver and kidney (186, 196). This mode of action may also underlie the ability of FB1 to act as a promoter of liver tumors or preneoplastic lesions by DNA reactive carcinogens such as aflatoxin B1, diethylnitrosamine, 7,12-dimethylbenz[a]anthracene and N-methyl-N′-nitro-nitrosoguanidine (199).

p53: A transcriptomic analysis using Caco-2 and HepG2 cells and analysis of the expression profiles for functional networks (200) has suggested that TP53 is considerably down-regulated. The authors speculate that lower TP53 expression might partially explain why the dosages of FB1 that were used in the studies did not cause extensive cell death, and it might also increase susceptibility to cancer. However, similar hepatic adenomas and cholangiomas have been seen in p53+/- and p53+/+ mice (106), so the role of p53 in cancers induced by fumonisins is uncertain.

Autophagy/Mitophagy: In a study using monkey kidney MARC-145 cells as a model for fumonisin-induced nephrotoxicity, FB1 was found to induce caspase-independent programmed cell death accompanied with autophagy. These effects were concluded to be via disruption of SL biosynthesis because they were blocked by myriocin (23). In mice, FB1 induced ER stress and activated apoptosis and autophagy in the colon (169).

In HepG2 cells, FB1 induced autophagy, but suppression of autophagy with 3-methyladenine enhanced apoptosis; therefore, autophagy activation might help protect the liver (170).

ER stress

Induction of ER stress: FB1 increased the expression of PERK, IRE1-α, and LC3I/II in HepG2 cells, which are markers of ER stress and autophagy (170).

Inhibition of ER stress: FB1 inhibited the induction of ER stress and the killing of two cancer cell lines (PC-3 and DU-145 cells) mediated by Ad.mda-7-triggered Cer elevation (201).

Altered mitochondrial function

FB1 has been reported to inhibit mitochondrial complex I, which leads to a decrease in the rate of mitochondrial and cellular respiration, depolarization of the mitochondrial membrane, induction of reactive oxygen species (ROS) production in mitochondria and deregulation of calcium signaling (202). This might be due to elevated sphinganine and reduced CerS activity because that has been shown to inhibit mitochondrial complex IV activity and increase ROS generation (203). Sphinganine also inhibits cytochrome oxidase activity in isolated mitochondria (204).

ROS production/Oxidative stress

Oxidative stress has been frequently proposed as a biochemical mechanism for fumonisin toxicity in vitro and in vivo (5). Elevation of reactive oxygen species (ROS) by fumonisins (205) could also contribute to the observed increase in lipid peroxidation(206) . There is an extensive literature on disruption of mitochondrial electron transport and other mitochondrial processes by SphB, SphB 1-phosphates and (DH)Cer (155). In addition, 1-deoxy-SphB interfere with mitochondrial functions because they concentrate in mitochondria (207). Interestingly, it has been reported that FB1 inhibits mitochondrial CerS activity with Sa but not So (163), which raises the possibility that this organelle gets a one-two punch because because it might “capture” both Sa and So, but only convert the latter to Cer.

Altered growth control/survival regulation

Cell cycle regulation: FB1 has been reported to affect multiple cell-cycle regulatory elements in cells where they are growth suppressive, such as CV-1 cells (208). These include induction of retinoblastoma (Rb) dephosphorylation (208), repression of Cyclin dependent kinase 2 (CDK2) activity and reduction of cyclin E protein levels, and induction of the CDK inhibitors p21(Waf1/Cip1), p27(Kip1), and p57(Kip2) (100). As noted earlier, Rb dephosphorylation is induced by treatment of cells with SphB (68), therefore, these might be consequences of the elevated SphB during FB1 treatment.

In vitro treatment of swine peripheral blood mononuclear cells with FB1 decreased proliferation with an increased percentage of cells blocked in G0/G1 phase of the cell cycle (209).

AKT: FB1 has been found to activate Akt to inhibit GSK-3β activity and stabilize cyclin D1 in livers of treated rats (210). AKT activation was also found in a "transcriptome and kinome" analysis of pigs fed FB1 (43).

Telomerase activity: There has also been noted that FB1 changes telomerase activity in mouse liver (211), which might involve SL metabolism because Cer has been found to reduce telomerase activity, and this is blocked by myriocin or FB1 (212, 213). The reduction of telomerase activity by Cer involves deacetylation of Sp3 and histone H3 at an hTERT promoter site, thereby preventing the transcription of hTERT (via HDAC1) (214).

Altered gene expression

Early studies of gene expression changes induced by FB1 found repression of PKC and stimulation of cyclic AMP response elements (16), which could be due to inhibition of PKC by Sa (12-14). Follow-up studies found that FB1 also repressed the activity of cyclin-dependent kinase 2 and induced expression of the CDK inhibitors p21(Waf1/Cip1), p27(Kip1) and p57(Kip2) in monkey kidney cells (CV-1) and a PCR-based subtraction approach (100) added eight more genes to the list.

Larger comparisons using Caco-2 and HepG2 cells and qRT-PCR-based gene profiler arrays found relationships between the differentially expressed genes that suggested activation of the NFκβ, ERK1/2, JAK, MAP2K1/2, Toll-like receptor signaling and activation of regulators of apoptosis and T lymphocytes (200). These are pathways often associated with SL signaling (10). As noted above, the analysis also suggested that TP53 was down regulated.

In pigs fed a control or FB1-(10 mg/kg bw)-contaminated diet for 4 weeks (43), the Sa/So ratio was elevated, as expected) and a "transcriptome and kinome" analysis of liver and intestine using microarrays for gene expression and peptide arrays for kinome analysis. The gene expression analysis found that the FB1-exposed animals had 49 up- and 74 down-regulated genes in the jejunum and 31 up- and 42 down-

regulated genes in the liver. The kinome analysis indicated that jejunum had 45 proteins with increased and 45 with decreased phosphorylation status; liver had 26 and 33, respectively. Analysis of the pathways affected by FB1 revealed that many were related to PI3K-AKT signaling, which they noted to be of particular interest because SL/Cer signaling pathways are known to modulate PI3K-AKT signaling cascades. They also noted that this could explain other pathways that were found to be affected by FB1 in their study, such as integrin-mediated cell-matrix adhesion and inflammation.

Cer (specifically C16-Cer) was recently found to be a regulator of transcription factor SNAI2 when shRNA-mediated knockdown of CerS 5 or CerS6 increased expression of SNAI2 (and restoration of C16-Cer suppressed SNAI2 expression) (51). SNAI2 is a member of the Snail superfamily of C2H2-type zinc finger transcription factors that is one of the transcriptional regulators of the epithelial to mesenchymal transition (EMT) that has important roles in development and cancer. In contrast to the suppression of SNAI2 by C16-Cer, S1P has been reported to activate SNAI2 via S1PR2 and S1PR3 receptors of MCF7 breast cancer cells to enhance cell invasion (50). Interestingly, over half of the renal tubule carcinomas in male rats from the two year feeding study were classified as a rare highly malignant variant (186).

Some effects on gene expression could also be due to altered epigenetic control (as discussed below).

Altered epigenetic regulation

Fumonisins have been found to alter multiple types of epigenetic regulation, including DNA methylation, histone modifications (methylation, acetylation and phosphorylation), and miRNAs (5, 215).

DNA methylation: FB1 has been reported both to increase and decrease DNA methylation depending on the type of cells and treatment conditions (5, 215). In rat C6 glioma cells, at levels of FB1 that caused some cytotoxicity, hypermethylation of the DNA was observed (216), but in HepG2 cells, cytotoxic doses of FB1 induced global DNA hypomethylation and three DNA methyltransferases were noted to have decreased activity (DNMT1, DNMT3A and DNMT3B) and one demethylase (MBD2) elevated activity (217). In another study, clone 9 rat liver cells did not display global

hypomethylation, but specific DNA regions had increased (c-myc and VHL genes) or decreased (*p*16 and *p*15 genes) CpG promoter methylation (218); a rat kidney epithelial cell line (NRK-52E) was also examined and showed a different pattern.

Histone modifications: Global histone modifications were found in NRK-52E cells exposed to FB1 with increased levels of H3K9me2/me3 but decreased H4K20me3 and H3K9ac (219). FB1 has been found to elevate Sa and Sa1P in the nucleus, and to decrease HDAC activity and increase histone acetylation at H2BK12, H3K9, H3K18, and H3K23 (98). An epigenetics study (220) of rats given a combination of FB1 and a methyl-deficient diet found that inclusion of FB1 had variable effects on H4K16Ac but decreased H4K20me3 and increased H3K9me3. All together, these studies indicate that elevation of nuclear Sa1P by fumonisin can cause a decrease in HDAC activity and increased histone acetylation, and possibly might account for the altered methylation.

Histone phosphorylation has also been noted to be affected by FB1 in HepG2 cells at high concentrations (200 μM) (221). FB1 caused a significant decrease in phosphorylated γ-H2AX, a histone variant H2AX that is an early cellular response to the induction of DNA double-strand breaks.

Altered cell-cell interactions

SL and GSL play multiple roles in cell-cell interactions. GSL mediate cell-cell recognition by binding to complementary glycan binding proteins (lectins) on apposing cells to mediate cell-cell adhesion and to control intracellular signaling pathways (222). S1P and S1P receptors also control dynamic processes such as the generation of lamellipodia, resealing of endothelial gaps, maintenance of barrier integrity and other endothelial barrier functions (223, 224).

There is evidence that disrupted cell-cell interactions are involved in fumonisin-induced farm animal diseases. For example, horses treated using intravenous administration of pure FB1 developed the neurological disease known as equine leucoencephalomalcia (ELEM) (225). It was concluded that the cause of the brain lesions was vasogenic cerebral edema and brainstem or cerebral herniation into the vertebral canal. Vasogenic edema results from a breakdown in the integrity of the

blood–brain barrier tight endothelial junctions. Analysis of the serum from the horses revealed significantly elevated Sa and Sa1P (226).

FB1 has been found to reduce barrier function using endothelial cells from porcine pulmonary arteries, where FB1 doubled the rate of albumin transfer across endothelial cell monolayers under conditions where there was no evidence for loss of cell viability (227). FB1 also reduced barrier function measured by transepithelial electrical resistance in IPEC-1 cells, a porcine intestinal epithelial cell line, below its cytotoxic dose (228). Disruption of barrior integrity in the intestines is a potential cause of malabsorption and enteric disease in humans and has been proposed to contribute to growth retardation in children in Tanzania (229). Several studies have shown a significant correlation between urinary or dietary fumonisin levels and growth retardation in children in Tanzania (230). The levels of urinary fumonisin in Tanzanian children is similar to the levels of urinary fumonisin in Guatemalan women sufficient to result in elevated levels of Sa1P and the Sa1P/So1P ratio in blood (231).

FB1 has also been shown to inhibits gap-junction intercellular communication using a scrape-loading dye transfer method and clone-9 rat liver cells (CRL-1439), a rat liver epithelial cell line (232). Interestingly, CerS2 ko mice also displayed compromised gap junction function (233) that was shown to be due to mislocalization of a key gap junction protein, connexin 32, resulting in accelerated rates of its lysosomal degradation. Furthermore, as a result of the reduced gap junction function, the mice were resistant to acetaminophen-induced hepatotoxicity.

Disrupted developmental regulation

Birth defects were not among the diseases initially linked to fumonisins, but the provocative evidence suggesting that they might be contributors to neural tube defects (NTD), in particular, was summarized in a workshop proceeding published in 2004 entitled: “Fumonisins disrupt sphingolipid metabolism, folate transport, and neural tube development in embryo culture and in vivo: a potential risk factor for human neural tube defects among populations consuming fumonisin-contaminated maize” (234). The connections—some of which were works-in-progress that weren’t published until later--were that a) depletion of membrane SL in cells in culture by fumonisins interfered with

the function of the folate-binding protein (human folate receptor alpha) that is responsible for folate transport, and defective folate transport (or dietary availability) was strongly associated with neutral tube defects (126); b) fumonisins cause neural tube and craniofacial defects in mouse embryos in culture, and many of the effects could be prevented by supplemental folic acid (235); c) studies in LMBc mice found that fumonisin exposure in utero increases the frequency of developmental defects and administration of folate or a complex sphingolipid is preventive (236); and d) high incidences of NTD occur in some regions of the world where substantial consumption of fumonisins has been documented or plausibly suggested (Guatemala, South Africa, and China) (3) and a recent study of NTD in border counties of Texas found a significant association between NTD and consumption of tortillas during the first trimester (237).

Subsequent studies have shown effects on growth and birth defects in a range of species) (230, 238). Additional support for the possible involvement of fumonisin and CerS inhibition in developmental defects include: a) a dose-dependent correlation between urinary FB1 and blood levels of Sa1P and the Sa1P/So1P ratio in humans consuming maize-based foods and living in high exposure communities in Guatemala (231); and b) new evidence linking exposure to plausible mechanisms for altered developmental gene expression, e.g., that FB1 treatment of mouse embryonic fibroblasts caused elevation of nuclear SphB1P and decreased histone deacetylase activity (98). The finding of an NTD (exencephaly) in embryos from SphK1/SphK2 double-knockout mice (56) further established that disruption of SL signaling during fetal development can cause NTD.

Altered immune function

It has been noted (239) that since fumonisins alter multiple SL that are involved in immune cell regulation (e.g.,Cer, Cer1P, So and S1P), they would be expected to also affect immune cell function. Some of the effects that have been noted are alterations in CD3, CD4, CD8, CD45, and other T lymphocyte surface antigen expression, lymphocyte subpopulation balance, and DNA synthesis, and suppression of the response to T-dependent antigens in vivo (239).

Some of the early studies in mice demonstrated that fumonisin injection altered SM and Cer amounts in thymus of mice and decreased the receptor CD3 expression on the surface of thymus cells "in vitro" and "in vivo (240). Other studies (241) that measured functional immune parameters (the number of plaque-forming cells) found both immunosuppression and immunostimulation, depending on the dosing protocol. In addition, there was evidence of an antigenic response to FB1.

In a long-term feeding study (26 weeks) (106), mice fed a diet with 150 mg FB1/kg tended to have higher WBC, lymphocytes, T lymphocytes, Th and Tc lymphocytes, B lymphocytes, NK cells, plasma IgA and IgM than the controls; IgG levels were notsignificantly affected. This study also analyzed each category of elevated SphB in liver and Sa and Sa1P were elevated 7.5- and 5.5-fold, respectively; 1-dSa was elevated 4 to 9-fold; and So was only elevated 1.2 to 1.5-fold (S1P was not detected in liver but was detected at high levels in kidney, as were Sa and Sa1P). Fumonisins have also been found to alter the function of murine bone marrow-derived dendritic cells (242).

Studies of rats (243) noted that FB1 reduced thymus weight, thymic necrosis and elevated serum immunoglobulin M and phagocytic cell numbers, and (244) stimulated nitric oxide production by macrophages and stimulated T cell proliferation. Intestinal immunity has been shown to be affected in pigs in a manner that might contribute to the increased intestinal colonization by pathogenic *Escherichia coli* (228, 245) and shift in microflora balance (246).

Cancer

The mechanism of FB1 carcinogenesis is not fully understood from the studies of cells in culture and rodents (the species where the carcinogenicity of FB1 has been conclusively established), however, there are many clues. It does not appear that fumonisins directly interact with DNA, nor that they are metabolized into a DNA-reactive compound (199); however, FB1 has been shown to induce a significant increase in 8-oxo-2'-deoxyguanosine (8-OH-dG), an indicator of oxidative stress, and DNA fragmentation in C6 glioma cells and mouse embryonic fibroblasts (MEF cells) (247). Since p53 heterozygosity did not influence the type and incidence of liver tumors

observed in mice with long-term FB1 exposure (106), it was suggested that DNA damage occurs downstream of the primary effects of FB1 on the cells.

As discussed throughout this Supplement and a previous review (3), fumonisins alter many cell regulatory mechanisms that are known to be important in cancer, such as increased amounts of mitogenic and anti-apoptotic SphB1P as well as reduced amounts of pro-apoptotic Cer, increased signals for EMT and cell migration, altered immune function, and others.

Concluding comments

Fumonisin inhibition of CerS results in changes in the pool size of many highly biologically active and structurally important sphingolipids, produces (from SphB1P degradation) precursors to other important lipids, and affects the metabolism and signaling of bioactive lipids in other lipid categories. Additional complexity arises from the fact that the downstream molecular targets and physiological consequences of the changes in the concentration of these lipid pools are undoubtedly time and dose-dependent, and include some effects that occur secondary to the responses initiated by the primary targets. Thus, the inhibition of CerS creates a perfect storm of perturbed sphingolipid metabolism, signaling and disease.

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