FOOD SAFETY

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Review (Invited)

Fumonisin Toxicity and Mechanism of Action: Overview and Current Perspectives

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Fumonisins are mycotoxins produced predominantly by *Fusarium verticillioides* and *F. proliferatum*. They contaminate maize and maize-based foods throughout the world. Fumonisin B_1 is the most common. It causes species-specific toxicities in laboratory and farm animals including liver and kidney cancer in rodents. Inhibition of ceramide synthase and disruption of sphingolipid metabolism is the non-genotoxic mechanism underlying its toxicological and carcinogenic effects. The extent to which fumonisin B_1 or other fumonisins impact human health remains poorly understood although epidemiological and experimental evidence implicate them as a risk factor for esophageal cancer and neural tube defects in populations consuming large amounts of contaminated maize-based foods. Selected toxicological investigations providing evidence for the above and serving as a basis for applied studies to better understand the extent of human exposure and potential risk are reviewed. The latter includes the use of kidney toxicity in rats as a bioassay showing that alkaline cooking (nixtamalization, the traditional method for making masa and tortillas) and extrusion effectively reduce the toxicity of fumonisin-contaminated maize and the development of robust exposure biomarkers for use in epidemiological studies. Future initiatives to better understand the relationship between fumonisins and human health should emphasize validation of biomarkers, such as urinary fumonisin B_1 concentration, as well as comparative studies to determine which animal models are most relevant to humans.

Key words: fumonisins, toxicity, mechanism of action, biomarkers

Introduction

Fumonisins B_1 and B_2 (**Fig. 1**) were first isolated from *Fusarium verticillioides* (previously *F. moniliforme* Sheldon) and their chemical structures reported by Gelderblom and colleagues[1] in 1988. Their publication was a milestone in mycotoxin research as, for the first time, a mycotoxin, in this case fumonisin B_1 (FB_1), could be shown to cause the toxicities associated with *F. verticillioides* or *F. proliferatum* including liver toxicity, cancer promotion and cancer in rats[1,2], leukoencephalomalacia in *Equines*[3], and pulmonary edema in swine[4].

Subsequent surveys have revealed that FB_1 and, to a lesser extent, fumonisins B_2 (FB_2) and B_3 (FB_3) are widespread natural contaminants of maize, maize-based foods and animal feed[5–7]. Fumonisins have occasionally been found in other commodities, mostly at low levels[7], and FB_2 has been found to be produced by some *Aspergillus niger* isolates[8]. Nonetheless, the potential risks of fumonisins to animal and human health are associated almost exclusively with *F. verticillioides* or *F. proliferatum*-contaminated maize. More than 30 fumonisin and modified fumonisins (**Fig. 1**) have

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Abbreviations: FB_1, fumonisin B_1; HFB_1, hydrolyzed fumonisin B_1; FB_1, fumonisin B_2; NDF-FB_1, *N*-(deoxy-D-fructos-1-yl)-FB_1; NCM-FB_1, *N*-(carboxymethyl)-FB_1; Sa/So, sphinganine/sphingosine ratio; NTD, neural tube defect

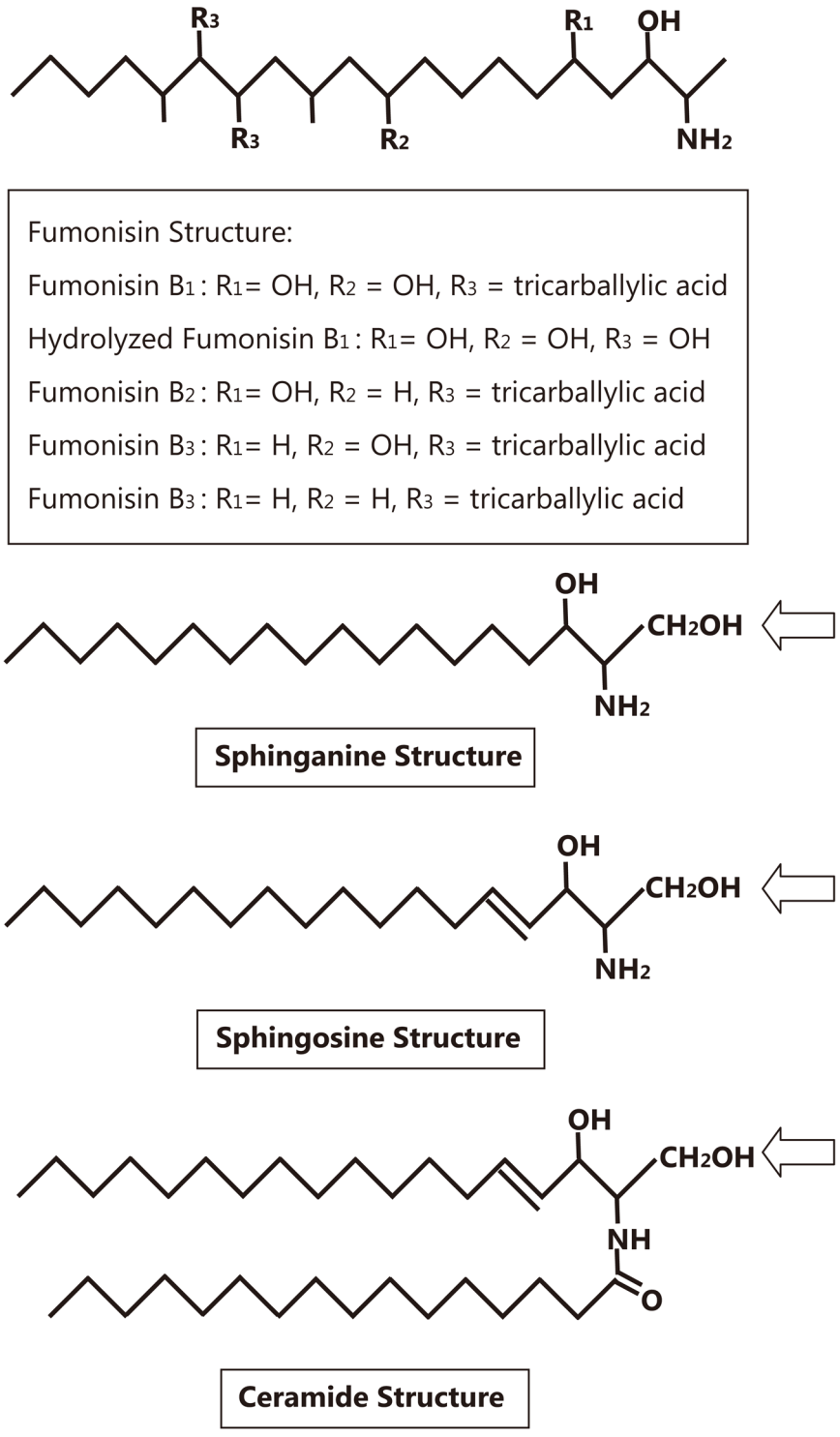

Fig. 1. Basic structure of fumonisins (top), sphinganine and sphingosine (middle) and ceramide (bottom). Fumonsins B_2 and B_3 can be hydrolyzed as shown for fumonisin B_1. Starches, sugars or other food matrix components can interact with the amino and tricarballylic acid groups. *N*-acylation of sphinganine produces dihydroceramide and double bond insertion between $C_4 - C_5$ produces ceramide. Sphingoid bases with a hydroxyl at C_1 can be phosphorylated to their 1-phosphate metabolites (arrow). Ceramide and dihydroceramide are converted to sphingomyelin and complex sphingolipids by additions to C_1 of its sphinganine or sphingosine moiety (arrow).

been found in maize or culture materials (maize fermented with fumonisin-producing fungi under controlled conditions) and the list is expected to continue growing[6,9,10]. FB_1 is however the most significant from a toxicological standpoint and most thoroughly studied due to its prevalence and biological effects.

Human exposure to fumonisins and their derivatives such as the hydrolyzed species that form under alkaline cooking conditions vary considerably depending upon geographical region and dietary custom[5,6]. Exposures are generally low in the industrialized countries of Western Europe, North America, and Japan but are higher and often exceed the provisional maximum tolerated daily intake of 2 μg/kg body weight established by the WHO/FAO Joint Expert Committee on Food Additives and Contaminants (JECFA) in areas of Africa, China, Central America and elsewhere[5,6]. A definite relationship between FB_1 or other fumonisins and human disease has not been established, but on the basis of observation and epidemiological studies they have been implicated as possible risk factors for cancer (esophagus, liver)[5,6,11] and birth (neural tube) defects[12,13] in populations depending on maize as a major food source. Additive or synergistic interactions with other mycotoxins such as aflatoxin cannot be ruled out[5,6,14,15].

Fumonisins became the subject of intense, multidisciplinary and multinational research activity following their discovery and comprehensive reviews of the findings are available[5,6]. Initial research by toxicologists in the USDA Agricultural Research Service (ARS) and their collaborators focused on defining the organ-specific effects of naturally contaminated maize, culture materials and purified FB_1 in rodents and farm animals, on acquiring dose-response data, and on determining fumonisin's mechanism of action. Subsequent areas of emphasis have been investigating the teratogenic potential of FB_1 and its hydrolyzed form (HFB_1) in mouse models, applied toxicological investigations to explore the efficacy of cooking methods to reduce concentrations and toxicity of fumonisins in maize products, and developing mechanism-based biomarkers for use in epidemiological studies. An overview of previous and current research initiatives by ARS toxicologists and other research groups in these areas follows.

Characterization of Liver and Kidney Toxicity

Rodent bioassays of naturally contaminated maize, culture materials and purified FB_1 confirmed that fumonisins were responsible for the hepatotoxicity of *F. verticillioides*-contaminated maize and culture material. They further established the kidney as a sensitive target organ in rats and explored dose-response in rats and mice.

Naturally Contaminated Maize and Culture Material

Two batches of maize (CS-1 and CS-2) associated with an outbreak of leukoencephalomalacia were fed to male Sprague-Dawley rats for four weeks. Hepatic lesions and serum chemistry findings[16] were consistent with those found in rats fed *F. verticillioides* culture materials[1,17]. They were also considered consistent with the (expected) early stages of the pathology described by Wilson *et al.* [18] that included neoplastic nodules, adenofibrosis and cholangiocarcinomas in the livers of rats fed fungal (*F. moniliforme*)-contaminated maize for 18 to 25 weeks.

Renal tubule lesions were well developed and, like liver lesions, simultaneously exhibited apoptotic, regenerative, and other features later recognized to be typical features of FB_1 nephrotoxicity[19]. Both hepato- and nephrotoxic effects were more pronounced in rats fed the more highly contaminated CS-1: quantification by liquid chromatography-selected ion mass spectrometry (LC-SIMS) found 150 ppm FB_1 therein and 20 ppm in the CS-2[20]. FB_2 was not quantified.

Diets prepared from *F. verticillioides* culture material, water or chloroform:methanol extracts of the culture material, the extracted culture material residues, or reconstituted combinations of extract plus residue were fed to male rats for four weeks[17]. The diets made from the unextracted culture material, water extracts of the culture material, and the residue after extraction with chloroform:methanol were hepatotoxic and gas chromatographic-mass spectrometric (GC-MS) analysis revealed 93 to 139 ppm FB_1 and 82 to 147 ppm FB_2 therein. The remaining diets were not hepatotoxic and contained $\leq$ 22 ppm FB_1 and $\leq$65 ppm FB_2. The reason why FB_1 concentrations of approximately 20 ppm in naturally contaminated maize (CS-2) were toxic[16] but were not toxic in the culture material fractionation experiment[17] was possibly due to differences in the analytical methods used (LC-SIMS versus GC-MS), diet FB_2 concentrations (not quantified in CS-1 and CS-2), or poor nutritional quality of the contaminated CS-1 and CS-2 maize.

The effects of *F. verticillioides* in mice (Balb/c strain) were then investigated by feeding diets amended with the culture material to provide 99 ppm FB_1 for four weeks[21]. Weight gain was reduced compared to mice fed a control diet formulated with equivalent weights of uncontaminated maize. Serum chemical indications of hepatotoxicity (increased transaminase and alkaline phosphatase activities and increased bilirubin concentration) and liver lesions were present

in all mice fed the culture material. Lesions were similar to those that occur in rats, with scattered apoptosis and foci of megalocytic hepatocytes. The apoptotic effect in liver was reproduced and a minor amount of apoptosis in the kidney tubules was also induced in another experiment by administering FB_1 by subcutaneous injection to mice over a five-day period[22].

Dose-response of FB_1

FB_1 purified from *F. verticillioides* culture material was fed to male and female Sprague-Dawley rats at concentrations of 0, 15, 50 or 150 ppm FB_1 for four weeks[19]. A no observed effect level (NOEL) for nephrotoxicity in males was not established as lesions and lipid metabolic effects (elevated sphinganine and sphingosine concentrations, see sections on "Sphingolipid Metabolism" and "Mechanism" below) were found at ≥15 ppm. The NOEL for nephrotoxicity in females was 50 ppm when based on histopathology findings. A NOEL based on tissue kidney sphingolipid effects was not determined as retrospective analysis revealed elevated sphinganine and sphingosine concentrations and an elevated sphinganine to sphingosine (Sa/So) at 15 ppm[23]. The NOEL for hepatotoxicity was 50 ppm on the basis of the microscopic lesions: lesions were found in both sexes fed 150 ppm and were somewhat more advanced in females. Sphinganine concentration and Sa/So were significantly increased, however, in the liver of both sexes fed 50 ppm: a NOEL based on sphingolipid findings was therefore 15 ppm[23].

Diet FB_1 concentrations for the 13-week feeding study in F344 rats were 0, 1, 3, 9, 27 and 81 ppm[24]. The FB_1 diets provided calculated mean daily intakes of 0.07, 0.21 0.62, 1.92 and 5.66 µg/kg body weight for males and 0.08, 0.24, 0.73, 2.15 and 6.35 µg/kg body weight for females. Apoptotic kidney lesions and increased serum creatinine were found in 14 of 15 males fed 9 ppm and all males fed 27 or 81 ppm. The severity of the lesions increased in a dose-dependent manner: they were judged "minimal" at 9 ppm, "minimum to mild" at 27 ppm, and "mild" at 81 ppm. Females were significantly less sensitive to the nephrotoxic effects as "minimal to mild" kidney lesions were found only in eight of the 15 females fed 81 ppm and serum creatinine was increased only at this dose. No evidence of hepatotoxicity was found in either sex. The respective NOEL values for nephrotoxicity in males and females were 3 ppm (=0.21 µg/kg body weight per day) and 27 ppm (=2.15 µg/kg body weight per day). Calculated lowest observed effect levels (LOEL) were 9 ppm (0.62 µg/kg body weight per day) for males and 81 ppm (6.35 µg/kg body weight per day) for females.

When fed to $B6C3F_1$ mice, the 0, 1, 3, 9, 27 or 81 ppm FB_1 diets provided average daily FB_1 intakes of 0.30, 0.84, 2.44, 7.38, or 23.1 µg/kg body weight and 0.31, 1.00, 3.03, 9.71, or 28.9 µg/kg body weight to males and females, respectively. No effects were found in males. In females, toxicity was limited to mild hepatic lesions at 81 ppm and accumulation of minimal to minor amounts of pigment (consistent with ceroid) in adrenal cortex macrophages in two (of 15) females fed 27 ppm and all females fed 81 ppm. The NOEL for FB_1 in mice was therefore 27 ppm in females (=9.71 µg/kg body weight per day) and >81 ppm (>23.1 µg/kg body weight) in males. LOEL values in the 13 week studies in rats and mice were therefore 9 ppm (=0.62 µg/kg body weight; male rat kidney) and 81 ppm (28.9 µg/kg body weight; female mouse liver), respectively.

To further define dose-response and establish dietary levels for chronic studies, Tolleson *et al.* [25] fed graded concentrations of 99 to 484 ppm FB_1 to F344 rats and B6C3F1 mice for four weeks. Liver and kidney pathology in the rats was consistent with earlier findings. Dose-response for liver effects suggested that F344 males were less sensitive than their Sprague-Dawley counterparts: hepatotoxicity was found at ≥234 ppm in the F344 strain as opposed to 150 ppm in Sprague-Dawley males. Hepatotoxicity was found at ≥163 ppm in females, a level roughly equivalent to that found in the Sprague-Dawley strain. Nephrotoxicity occurred at ≥99 ppm in males but at ≥163 ppm in females, thus corroborating earlier results on relative sensitivities male and female kidneys[19,24].

In mice, the liver was the only organ identified as a target although others have described kidney tubule lesions as well[22,26]. The incidences of animals exhibiting apoptosis and other indications of hepatotoxicity were significantly increased in males fed 484 ppm (100%) and in females fed ≥99 ppm FB_1 (dose-related increase from 50% at 99 ppm to 100% at 234 and 484 ppm).

Carcinogenicity

The hepatocarcinogenicity of FB_1 was first demonstrated by Gelderblom *et al.* [2] in a study in which a diet containing 50 ppm FB_1 was fed to male BD IX rats. In a follow-up study of similar design, they found a dose-related induction of glutathione-S-transferase (placental form) positive nodules in livers of BD IX rats chronically fed 10 or 25 ppm FB_1, but no overt parenchymal or bile duct cancers at the lower dietary exposure levels[27].

Studies to further characterize carcinogenicity and establish dose-response were undertaken by the National Toxicology Program of the US Food and Drug Administration[28]. In these chronic bioassays, FB_1 was fed to male and female F344 rats for two years at concentrations of 0, 5, 15, 50, or 150 ppm to males and 0, 5, 15, 50, or 100 ppm to females. FB_1 was nephrocarcinogenic in male rats at ≥50 ppm. The combined incidences of tubule adenoma and carcinoma were 19 and 31% at the two highest doses and it is noteworthy that some neoplasms were of a rare and aggressive sarcomatous variant[29]. Metastases were found in the lung and lymphatic tissues. No other carcinogenic effects were found in either sex.

FB_1 was also fed to $B6C3F_1$ mice for two years at diet concentrations of 0, 5, 15, 80 or 150 ppm and 0, 5, 15, 50 or 80 ppm for males and females, respectively[28]. In contrast to rats, the male mice exhibited no carcinogenic effects whereas liver neoplasms were found in females. The combined incidence of hepatic adenomas and carcinomas increased in a dose-dependent manner from 40% (19/47) and 87% (39/45) at the two highest diet concentrations whereas incidences were 11% (0 ppm) or less in the other groups.

Hepatic adenomas (30 to 40% incidence per group, n =10/group), cholangiomas (10 to 20% incidence), oval cell proliferation (100% incidence) and foci of megalocytic hepatocytes (100% incidence) were induced in male p53 homozygous wild type and p53 heterozygous transgenic mice that had been fed diets amended with 150 ppm FB_1 for 26 weeks[30]. With the exception of megalocytosis, these effects were not evident at the lower diet concentrations tested (5 and 50 ppm FB_1). However, focal hepatic megalocytosis as well as apoptosis, necrosis, and mitosis were found in both homozygous and heterozygous animals: their incidence and severity were similar and increased in a dose-dependent manner.

Hepatic sphinganine concentrations were significantly increased in homozygous mice at ≥50 ppm FB_1 and in the heterozygous animals at 150 ppm. Sphinganine 1-phosphate was increased at 150 ppm and 1-deoxysphinganine (produced when alanine substitutes for serine in the first step of *de novo* biosynthesis) at ≥50 ppm FB_1 in both the homozygous and heterozygous mice[30]. The findings are consistent with a non-genotoxic mode of action mediated through disrupted sphingolipid metabolism and provided no evidence that p53 dependent pathways play a primary role in FB_1 carcinogenesis in mouse liver. They further suggest that any DNA damage that might occur is secondary to lipid metabolism disruption that results from ceramide synthase inhibition.

Sphingolipid Metabolism

FB_1 and other fumonisins having a free amino group at C_1 exert their biological activity by inhibiting ceramide synthases (sphingoid base *N*-acyltranferases), a family of enzymes catalyzing *N*-acylation of sphinganine, sphingosine, and other sphingoid bases to form ceramides, which are then further metabolized to more complex sphingolipids[31,32] (**Fig. 2**). Inhibition of ceramide synthase is competitive and results from the structural similarity of fumonisins to sphinganine and sphingosine (**Fig. 1**). Evidence for inhibition of ceramide synthase by FB_1 was first presented by Wang *et al.*[31], who found that FB_1: (a) inhibited incorporation of radiolabeled serine into sphingolipids of cultured hepatocytes while concurrently causing accumulation of sphinganine; (b) reduced the incorporation of radiolabled sphingosine into ceramide of hepatocytes; and (c) inhibited ceramide synthase activity in rat liver microsomes. Since then, inhibition of ceramide synthase by FB_1 and other fumonisins having a primary amino group has been demonstrated in multiple mammalian, avian, and piscine species as well as in plants[5,6,33,34]. Fumonisins not having a primary amino group in contrast did not inhibit ceramide synthase in mice[33].

Ceramide synthase inhibition *in vivo* leads to rapid accumulations of sphinganine, lesser accumulations of sphingosine, elevated Sa/So, accumulations of the 1-phosphate metabolites of sphinganine and sphingosine, and decreases of downstream complex sphingolipids (reviewed by Bulder *et al.*[6]). In mice exposed to FB_1 there are also dose-related increases in 1-deoxysphinganine, the sphingoid base most similar in structure to FB_1, and 1-deoxysphingosine together with decreases in complex sphingolipids containing these deoxy sphingoid bases in liver and, to a lesser extent, in other tissues[6,35]. Interestingly, deoxysphinganine is not detected in liver or kidney of rats treated with FB_1 and the levels of 1-deoxysphinganine accumulation in mouse liver and kidney are much greater than the accumulation of sphingoid base 1-phosphates[30]. The differences among species in accumulation of specific sphingoid bases and sphingoid base metabolites may contribute to the species specific targets of FB_1. The accumulation of sphingoid bases in response to fumonisin exposure shows organ-specific differences in rats: for example, accumulation of sphinganine 1-phosphate can equal or exceed the accumulation of sphinganine in rat kidney but very little sphinganine 1-phosphate accumulates in rat liver[36]. In plants, inhibition of ceramide synthase results in accumulation of primarily phytosphingosine and phytosphingosine

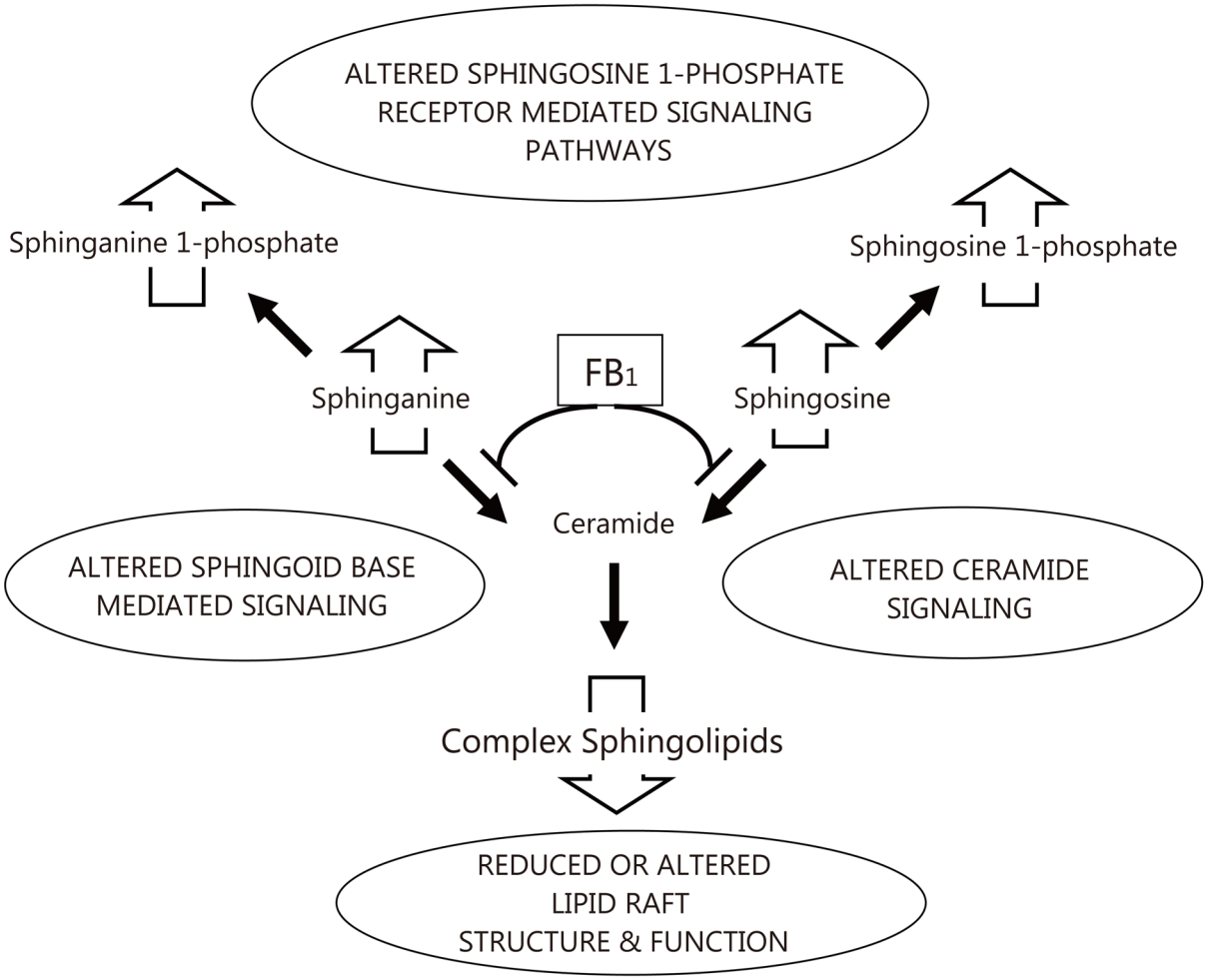

Fig. 2. Simplified depiction of the *de novo* sphingolipid biosynthetic pathway (solid black arrows indicate flow of metabolites through the pathway) and fumonisin B_1 (FB_1) mechanism of action. FB_1 disrupts the synthesis of ceramide from fatty acids and sphinganine or sphingosine by competitive inhibition of ceramide synthase. Consequently, concentrations of upstream metabolites increase (open block up arrows) and downstream metabolites decrease (open block down arrows). Signaling and other functions affected by altered concentrations of these metabolites are briefly summarized (ovals). Other consequences include disruption of various cytokine signaling pathways, altered lipid metabolism with changes in cell membrane composition, and oxidative damage (see text).

1-phosphate and lesser amounts of sphinganine and sphinganine 1-phosphate[34]. Sphingosine is uncommon in plant tissue just as phytosphingosine is uncommon in animal tissues.

These changes, particularly elevated sphinganine concentration or Sa/So in tissues, serum and urine serve as useful biomarkers of fumonisin exposure in animal experiments. Specifically, tight correlations between elevated sphinganine or increased Sa/So and the onset of clinical signs of toxicity or fumonisin-specific lesions in target tissues of farm or experimental animals have been repeatedly demonstrated: sphingolipid effects occur earlier during time-course experiments or at lower doses than the emergence of clinical or histopathological effects. This is illustrated by the following examples in two species.

Rats

Male rats were fed diets amended with *F. verticillioides* culture material to provide 14 or 89 ppm total fumonisins (FB_1 + FB_2 + FB_3 at a ratio of 100:45:10), or a control diet (found to contain 1 ppm fumonisins) for up to ten days[36]. The animals were examined at intervals to determine the time course of kidney and liver lesion development, tissue and serum sphingolipid alterations, and kidney and liver accumulations of fumonisins. The onset and severity of kidney pathology were both time- and dose-dependent. Apoptosis of proximal tubule epithelial cells and other indications of

toxicity were first noted in the high-level 89 ppm group after three days and in all animals fed 14 or 89 ppm fumonisins beginning at five days.

In contrast to histopathological changes, significant dose-related elevations in kidney sphinganine concentration were found after only one day of exposure in both the 14 and 89 ppm groups. Sphinganine 1-phosphate concentration was only slightly elevated after one day in the 89 ppm group. However, it continued to increase thereafter and reached a level about equal to that (nmol/g tissue basis) of sphinganine by three days. Lesser, but continuously rising concentrations of sphinganine 1-phosphate were found in the 14 ppm group beginning at three days. Kidney sphingosine and sphingosine 1-phosphate concentrations rose only slightly, but did so in a time- and dose-dependent manner. In the high-dose group, sphinganine, sphinganine 1-phosphate, sphingosine and sphingosine 1-phosphate concentrations reached their maxima by five days, the same time at which histopathological effects were most severe. Liver histopathology findings were subtle and relatively minor (compared to kidney) alterations in sphingolipid concentrations were found. It is noteworthy in this regard that (a) FB_1 accumulated in both organs in a time- and dose-dependent manner and (b) FB_1 levels in the kidney were ten-fold (high-dose) or more (mid-dose) higher than in liver after three days.

Ponies

Studies in ponies given fumonisin contaminated feed serve as another example illustrating the time-course relationship between sphingolipid metabolism disruption and disease, in this case hepatopathy and leukoencephalomalacia. The latter, often abbreviated in the literature as ELEM, is caused by FB_1[3], is usually of rapid onset, and involves progressive clinical signs of neurotoxicity and death. It is characterized *post mortem* by the presence of focal or multifocal liquifactive, hemorrhagic lesions in the white matter of the brain and spinal cord.

In the first of these investigations[37], one of four ponies fed 44 ppm FB_1 exhibited clinical signs of leukoencephalomalacia (not confirmed) and died within 10 days of liver failure, a second was euthanized after 45 days and was confirmed to have died of leukoencephalomalacia. Serum sphinganine concentration and Sa/So increased and complex sphingolipids decreased within a few days after these ponies were given the contaminated feed. In one case, the elevated serum sphingoid bases decreased and approached normal values as the animal reduced its feed consumption only to rise a second time as the animal began increasing its feed intake. Serum aspartate aminotransaminase concentration was markedly elevated at day 10 in the animal dying of liver failure but not at earlier time points, indicating that the sphingolipid effects occurred prior to the onset of disease.

Another pony was fed 15 ppm FB_1 over a 130 day period and showed only a modest 1.5 to 2 fold increase in Sa/So. The ratio was reduced upon withdrawal of the feed and then alternately increased and decreased as more highly contaminated feed (22 ppm FB_1) was reintroduced and withdrawn until the animal died of leukoencephalomalia at day 241. Serum chemical indication of hepatic damage was first evident at day 231, at least one month after serum sphingolipid changes were detected. These findings corroborated the earlier observations that serum sphingolipid profiles change rapidly after ponies are exposed to FB_1 contaminated feed, are reversible when exposure is reduced, and precede the onset of clinical signs or overt leukoencephalomalacia. The rapid reversibility of sphingolipid effects in fumonisin-exposed animals has been confirmed in other species, notably rats[38] and mice[39].

In a second set of experiments[40], the effects of (predominantly) FB_2-producing and FB_3-producing *F. proliferatum* culture materials on the induction of hepatotoxicity, leukoencephalomalacia and sphingolipid concentrations of ponies were compared. The culture materials contributed 75 ppm of FB_2 or FB_3 (<3 ppm FB_1) to the experimental diets. Overt leukoencephalomalacia was found in one and focal liquifactive lesions found in a second pony that was fed the FB_2 contaminated feed. The third pony from the FB_2 group did not exhibit leukoencephalomalacia when examined at necropsy but, like the other two in the group, periodically showed clinical signs of neurotoxicity beginning 148 days after exposure began. Serum enzymes indicative of liver damage were elevated in this group as early as 34 days but periodically rose and fell for the remainder of the trial.

Elevated serum sphinganine concentrations and Sa/So were found in the ponies fed 75 ppm FB_2 as early as four days after introduction of the contaminated feed, well before the appearance of serum chemical or neurological signs of toxicity. At necropsy, liver and kidney sphinganine and Sa/So were also significantly elevated and complex sphingolipids significantly decreased in the ponies fed 75 ppm FB_2. Animals given the FB_3 contaminated feed neither developed leukoencephalomalacia nor showed serum chemical evidence of hepatic injury. Serum sphinganine concentrations and Sa/So increased in this group also, although this effect was slightly delayed (first being noted after 11 days) and the values were significantly lower than in the FB_2-fed group. Likewise, elevations of sphinganine and Sa/So in kidney and liver, while present, were less marked in ponies fed FB_3 relative to those given FB_2. These results confirmed the time-course

relationships between altered sphingolipid metabolism and the later development of clinical disease found in the first study and indicated that FB_2 was more toxic to horses than FB_3.

F. verticillioides culture materials producing FB_2 or FB_3 were equally nephro- and hepatotoxic when fed to rats for three weeks[38], suggesting some species-specific differences in their relative potencies to ponies and rats exist. As in ponies, the sphingolipid effects in the rats fed FB_2 or FB_3 contaminated ration were reversible upon withdrawal of the contaminated diet. The microscopic appearance of liver and kidney also returned to normal within three weeks of stopping exposures, further implicating disrupted sphingolipid metabolism as the key mechanistic event and demonstrating the potential usefulness of serum and tissue sphinganine concentration or Sa/So ratio as experimental biomarkers.

Mechanism

The weight of evidence indicates that inhibition of ceramide synthase and the consequences thereof are the mechanistic trigger for fumonisins toxicity (**Fig. 2**)[5,6]. Some of the consequences are summarized below.

The upstream intermediate, sphinganine, of the *de novo* pathway and to a lesser degree sphingosine, which is reincorporated into ceramide as part of the salvage pathway, both accumulate following enzyme inhibition and have pro-apoptotic properties. Sphinganine and sphingosine are therefore likely to be either directly or indirectly involved in inducing apoptosis of renal tubule cells and hepatocytes, the first microscopically detectable indication of exposure in rats and other species[5,6]. It has been further suggested that the pathogenesis of pulmonary edema in swine is mechanistically mediated, at least partly, through inhibition of L-type calcium channels in the heart caused by the accumulated sphingosine (or sphinganine), which then leads to left-sided cardiac insufficiency[7]. A similar phenomenon might also underlie cardiotoxicity in FB_1-exposed horses[7].

The accumulated sphingoid bases are phosphorylated by kinase enzymes to their 1-phosphate metabolites which also accumulate. These compounds, particularly sphingosine 1-phosphate and sphinganine 1-phosphate are biologically active by acting as a ligand for G-protein coupled cell surface receptors known as sphingosine 1-phosphate receptors (S1PR; formerly endothelial growth (Edg) receptors). These receptors are critical for extracellular signal transduction governing diverse processes including immunity, cell to cell adhesion, mitosis, regeneration and cell migration[5–7]. Interestingly, intravenous administration of sphingosine 1-phosphate to naïve rats reduced renal blood flow whereas blood flow was not affected if the animals were pretreated with pertussis toxin[41]. This suggests that ischemia triggered by binding of accumulated sphingosine 1-phosphate to S1PR might be mechanistically involved in the apoptotic effects of FB_1 in rat kidney. Evidence that signaling via S1P receptor-mediated events modulates the developmental effects of FB_1 in the LM/Bc mouse model are found in the section on "Reproduction and Development". The possible role of decreased complex sphingolipids downstream of ceramide synthase (**Fig. 2**) in mediating some aspects of fumonisin toxicity, including neural tube defect induction by FB_1 is also discussed under "Reproduction and Development".

The mechanism of FB_1 carcinogenesis in rodents is not fully understood as the series of events linking ceramide synthase inhibition and disrupted sphingolipid metabolism to kidney and liver toxicity and finally to tumorigenesis has not been elucidated. Results from studies on mechanistic aspects of FB_1 toxicity in rodent models have revealed a number of possibilities including: (a) increased mRNA expression of genes modulating apoptosis[42,43]; (b) increased expression of tumor necrosis α[42]; (c) increased expression of genes involved in mitosis or regulating cell cycle progression, particulary the G_1/S transition[42–44]; (d) oxidative stress and secondary damage to macromolecules[45]; (e) altered lipid biosynthesis and changes in the composition of lipids in cell membranes[46,47]; and (f) diet composition and nutritional status, especially protein content of the ration[48]. It is likely that a combination of the above and possibly other effects resulting from disrupted sphingolipid homeostasis and signaling is ultimately involved.

In any event, the preponderance of findings indicates that carcinogenicity involves non-genotoxic events that do not involve binding of FB_1 directly with DNA[5,6,30]. Rather, the mode of action involves progressive toxicity leading to an imbalance between apoptosis (including inhibition of removal of "damaged" cells through apoptosis) and compensatory regeneration.

Effect of Cooking

Extrusion

Extrusion is a versatile cooking method having widespread application in the food industry. It involves forcing dough through a heated nozzle using one rotating (single-) or two counter-rotating (twin-screw extrusion) screws. Extrusion has been shown to reduce fumonisin concentrations in maize products. However, the amount of reduction varies depending on the particular combination of recipe, moisture content, cooking time and temperature, nozzle configuration, type and rotation speed of the screw(s). Chemical analyses or *in vitro* testing might underestimate the toxic potential of extruded foods (or other cooked cereal products) due to the formation of uncharacterized degradation or fumonisin-matrix interaction products that provide a "reservoir" of undetectable, yet bioavailable, fumonisin species. *In vivo* bioassays in conjunction with detailed chemical analyses have been used to explore the effectiveness of single-screw and twin-screw extrusion methods to reduce toxicity.

Two batches of *F. verticillioides* fermented maize grits (designated FG1 and FG2; with 33 and 48 ppm FB_1 respectively) were divided into three portions each[49,50]. Two portions were single-screw extruded (160 °C; 60 rpm screw speed) either without (FG1-E, FG2-E) or with (FG1-EG, FG2-EG) the addition of 10% (w/w) glucose. The third portions were not cooked. Another batch of grits was spiked with 30 ppm FB_1, divided and processed similarly (SG, SG-E, SG-EG).

HPLC and LC-MS analyses determined FB_1 concentrations and mass balance of FB_1 species (nmol/g dry weight FB_1, HFB_1, and the FB_1-glucose interaction products *N*-(deoxy-$_D$-fructos-1-yl)-FB_1 (NDF-FB_1) and *N*-(carboxymethyl)-FB_1 (NCM-FB_1)) in the cooked grits[49]. Extrusion had a minor impact on FB_1, reducing its concentrations by only about 10 to 28%. Extrusion of the glucose supplemented grits however reduced FB_1 by 75 to 85%. Minor amounts of HFB_1 and NCM-FB_1 were found and only low levels of NDF-FB_1, <1.0 ppm, were detected in uncooked grits or grits extruded without glucose (SG-E, FG1-E, FG-2E). However, NDF-FB_1 was found at levels of 9.0 to 17.0 ppm in extruded-glucose supplemented grits (SG1-EG, FG-1EG, FG-2EG) and therefore made up 57 to 66% of the FB_1 species found (mass-balance basis) therein.

It is significant that mass balance-based estimations revealed incomplete recovery of FB_1 and its reaction products in the cooked products. Recoveries ranged from 65 to 82% after extrusion alone and 35 to 65% after extrusion with glucose, suggesting that significant amounts of FB_1 were converted to uncharacterized reaction product(s), especially when glucose was added to the grits. Taking this into consideration, total fumonisins (FB_1 species + FB_2 + FB_3) were reduced by 18 to 35% by extrusion (SG-E, FG-1E, FG-2E) and were further reduced, by 35 to 65%, by extrusion with glucose supplementation. Mass balance of FB_2 and FB_3 was not determined but their concentrations were reduced similarly to that of FB_1.

Equivalent weights (dry weight basis) of the uncooked, extruded or extruded-glucose supplemented grits were blended with standard rodent ration and fed to male rats for three weeks[50]. FB_1 concentrations (ppm) in the test diets were: FG1 = 13.3, FG1-E =9.7, FG1-EG =1.9, FG2 = 19.4, FG2-E =16.5, FG2-EG =3.8, SG1 = 12.7, SG1-E =11.5 and SG1-EG =3.3. Control groups were fed diets made with uncontaminated grits. With the exception of FG-1EG, the test diets caused decreased relative kidney weights and moderately severe apoptotic lesions. The FG1-EG diet (prepared with extruded-glucose supplemented fermented grits) was also nephrotoxic, but significantly less so, as it did not affect kidney weight and lesion severity was judged as only minimal to mild. In summary, single-screw extrusion with glucose supplementation reduced fumonisin concentration but its effectiveness to reduce toxicity was limited, likely by a combination of the initial mycotoxin concentration and other as yet undefined factors.

Twin-screw extrusion offers advantages over the single-screw process such as improved flexibility and more efficient mixing of the cereal dough. Therefore, its effect on fumonisins in two batches of fermented grits (Batch 1 = 10 ppm free FB_1; Batch 2 = 50 ppm free FB_1; wet weight basis; "free FB_1" being defined as FB_1 concentrations quantified using standard extraction, clean-up and analysis methods) was determined, with the assumption that the twin-screw process would more effectively reduce fumonisins[51]. The approach was similar to that described above but was modified to provide for quantification of both free and matrix-associated FB_1 (referred to as "masked", "hidden" or "bound" FB_1 in the literature; enhanced techniques are required for their extraction, clean-up and analysis[51]), and a more rigorous bioassay that included clinical chemistry, renal sphingolipid profile, and histopathology evaluations after three and eight-weeks of feeding[52]. Test diets (50% grits; w/w) were formulated on a wet weight (rather dry weight equivalent) basis.

Twin-screw extrusion reduced FB_1 concentrations by 63 (Batch 2-E) to 71 (Batch 2-E)% and glucose supplementation again enhanced reductions, by as much as 89 and 94% respectively. Matrix-associated FB_1 was not found before cooking but constituted 25 to 38% (nmol/g basis) of the total FB_1 species present after extrusion without or with glucose.

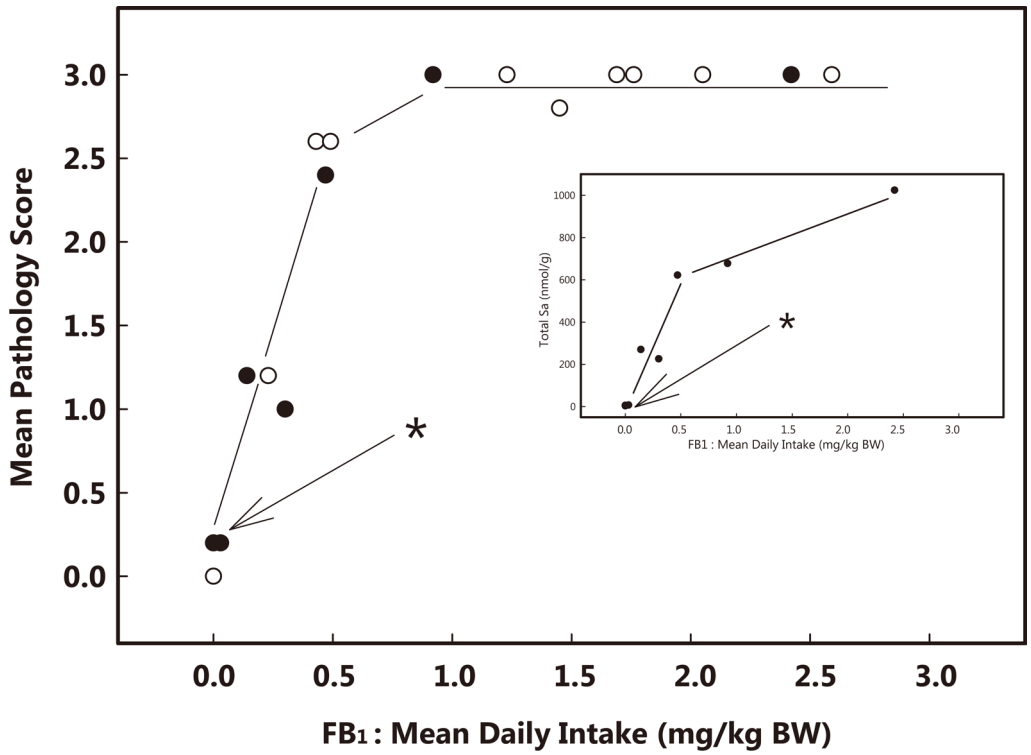

Fig. 3. Dose response comparing mean daily fumonisin intake and kidney pathology severity scores from studies on the effect of single-screw[49,50] (open circles) and twin-screw[51,52] (closed circles) extrusion on fumonisin toxicity. Pathology scores of 1, 2, and 3 respectively indicate kidney lesions of minimal, mild or moderate severity as defined in Voss *et al*. [50,52]. Inset shows dose-response for total kidney sphinganine (Sa) concentration (y-axis) versus mean daily intake in the two studies. Asterisk-arrow indicates scores and Sa of control groups and the group fed grits processed by twin-screw extrusion with glucose (FG2-EG, see text)[52].

The FB_1-matrix reaction products were not characterized but were probably, at least in part, the result of heat catalyzed interactions between the mycotoxin's tricarballylic side chains and matrix proteins or polysaccharides as described by Seiferlein and Humpf[53].

Only small amounts of NDF-FB_1 (<6%) were found in uncooked or grits extruded without glucose supplementation. Significant levels of this FB_1-glucose reaction product were, as expected, formed during extrusion with glucose: mass balance calculations revealed that NDF-FB_1 made up 36 to 38% of the total FB_1 species in Batches 1-EG and 2-EG. Low levels of HFB_1 were found in three of the four cooked grits preparations and contributed from 4 (Batch 2-EG) to 15 (Batch 1-E)% to the total FB_1 species. In contrast, HFB_1 accounted for ≤1% of the FB_1 species in the grits before extrusion.

Mass-balance of FB_1 in the cooked products was again incomplete. Total FB_1 species (free and matrix-associated FB_1 + HFB_1 + NDF-FB_1) were reduced by 35 (Batch 2-E) to 42 (Batch 1-E)% in extruded grits and by 69 (Batch 2-EG) to 80% (Batch 1-EG) in the extruded-glucose supplemented grits. This once more indicated that significant amounts of FB_1 in the grits had been converted to unknown and toxicologically uncharacterized reaction products by extrusion processing.

FB_1 concentrations (ppm) in the test diets formulated (50% w/w grits; wet weight basis) for the bioassay[52] were: Batch 1 = 4.9, Batch 1-E =1.4, Batch 1-EG =0.3, Batch 2 = 25, Batch 2-E =9.0, and Batch 2-EG =2.9. Toxicological findings were limited to the kidneys and consisted of the apoptotic tubule lesions and elevated concentrations of sphinganine, sphinganine 1-phosphate, sphingosine and sphingosine 1-phosphate that are characteristic of FB_1. Extrusion partially reversed these effects and glucose supplementation enhanced the effectiveness of extrusion in a FB_1 dose-dependent manner. As a result, additional but still partial protection was found in the group fed Batch 2-EG while no evidence of fumonisin exposure was found in rats fed diet made with the lesser contaminated (only 0.3 ppm FB_1) Batch 1-EG grits.

Kidney histopathology and sphingolipid effects in the bioassays of single screw[50] and twin screw[52] extruded grits were FB_1 dose (dose=calculated daily intake)-dependent (**Fig. 3**). The respective no observed adverse effect (NOAEL) and lowest observed adverse effect (LOAEL) levels based on 3-week kidney histopathology and sphingolipid results

from the twin-screw extrusion bioassay were 34 µg/kg body weight and 143 µg/kg body weight per day. Kidney lesions and sphingolipid effects were less pronounced at 8 weeks than at 3 weeks. This was likely due to the lower daily FB_1 intakes that were a consequence of the animals' weight gain during weeks 4 through 8 alone or in combination with some physiological adaption to exposure. As a result, lower NOAEL and LOAEL values of 25 and 103 µg/kg body weight were calculated on the basis of the toxicological findings at 8 weeks. Neither a NOAEL nor a LOAEL could be calculated from the results of the bioassay of single-screw extruded grits although the latter was ≤230 µg/kg body weight.

Most evidence indicates that NDF-FB_1 and HFB_1, the major FB_1 degradation products found in extruded grits, and NCM-FB_1 are significantly less toxic than FB_1[33,34] and therefore were not likely to have contributed significantly to the nephrotoxic effects of the grits. The potency of FB_2 and FB_3 are however not certain. FB_2 was toxic when given rats[44] but like HFB_1, NDF-FB_1 and NCM-FB_1, not when fed to mice[33]. Furthermore, when diets containing FB_1, FB_2 and FB_3 were fed to rats, accumulation of the latter two congeners in the kidney was disproportionately low, suggesting that their bioavailability is less than that of FB_1 or that there are other significant differences in their pharmacokinetic properties[36]. In any event, the extent to which FB_2, FB_3 and matrix-associated fumonisins contributed to toxicity is not known.

While the two bioassays of extruded grits offered no direct evidence for toxicity of matrix-associated fumonisins or unknown thermal degradation products, it is of interest that the NOAEL established in the twin-extrusion study was lower than those previously calculated on the basis of renal toxicity or carcinogenicity (≥200 µg/kg BW) of purified FB_1 in rats[5,6,28]. Detailed comparison of dose-responses from studies on extruded grits (using FB_1 concentration as marker for total fumonisins), on *F. verticillioides* culture materials, and on purified FB_1 led the WHO/FAO Joint Expert Committee on Food Additives and Contaminants to express the opinion that "…other toxins produced by *F. verticillioides* either add to or potentiate the toxicity of FB_1"[6]. Determining the degree to which this might occur is important as (a) up to 80% of the FB_1 in the raw grits was not accounted for after processing, (b) matrix-associated fumonisins can be present at relatively high levels in maize and foods[50,52,54], and (c) results of *in vitro* experiments suggest that matrix-associated fumonisins become bioavailable during digestion[55].

Nixtamalization

Nixtamalization is the traditional method for preparing masa from maize, which is then used to make tortillas and other food products. The process involves cooking maize in alkaline water, steeping, rinsing and then grinding. Tortillas and nixtamalized foods are diet staples in Mexico, Central America and among Hispanics in the United States and it is therefore important to understand the impact of alkaline cooking on mycotoxins.

Fumonisins are converted under alkaline conditions to their partially hydrolyzed ($PHFB_x$, lacking one of the tricaballylic groups) or hydrolyzed (HFB_x) forms, species lacking one or both of their tricarballylic acid groups (**Fig. 1**). The efficiency of this reaction, that is, the amount of fumonisin converted to partially or fully hydrolyzed species varies depending upon factors such as condition of the maize, fumonisin concentration, cooking time and recipe[56]. In one experiment for example, fried tortilla chips were made from batches of maize containing 0.2, 1.5, 1.9 or 47 ppm FB_1 (HPLC analysis) under conditions used by the snack food industry[57]. The process consisted of nixtamalization, rolling and cutting the masa, baking, and frying. FB_1 levels in the tortilla chips (wet weight basis) from the respective maize batches were 0.1, 0.3, 1.0 and 11 ppm, values corresponding to reductions of about 50 to 75%. FB_2 was similarly reduced. Cooking-steeping was clearly the critical step for reducing FB_1 and FB_2 as baking and frying had only a negligible effect. Follow-up LC-MS/SIMS analysis of the maize, masa and cooking-steeping liquid from two of the batches was done to estimate mass balance of FB_1, HBF_1, and $PHFB_1$. Thirty-four to 45% of the FB_1 in the raw maize remained in the masa. The ratio of FB_1:HFB_1:$PHFB_1$ in two batches of masa were 100:34:24 and 100:119:19 with the difference likely being a function of the 10-fold difference in the amounts of the FB_1 species (53 and 5.3 µmol/kg FB_1 + HFB_1 + $PHFB_1$) in the uncooked maize. It is noteworthy that a significant amount, 25 to 82% (mean =52%) of the mycotoxin in maize was recovered in the cooking-steeping liquid, mostly (>80%) as HFB_1.

Nixtamalization as practiced by Mayan households in Guatemala[58] reduced total fumonisin (FB_1 + FB_2 + FB_3 and their alkaline hydrolysis products) concentrations in tortillas by 50%. About equimolar amounts of FB_1 and HFB_1 were found in the tortillas and, as in the aforementioned experiments, almost all of the fumonisin found in the cooking-steeping and rinse liquids was HFB_1. Despite variability in recipes and processing times, significant reductions in FB_1 of 80% or more were achieved during masa production in small-scale commercial establishments in Texas[56]. Again, frying the masa to make tortillas yielded little to no further reduction. As discussed above, analytical evidence alone is insufficient to conclude that nixtamalization reduces the potential toxicity of masa products.

Bioassays comparing the effects of uncooked maize and masa and tortilla products were therefore conducted to address this issue. The initial *in vitro* experiments showed that the ceramide synthase inhibitory activity of fumonisins

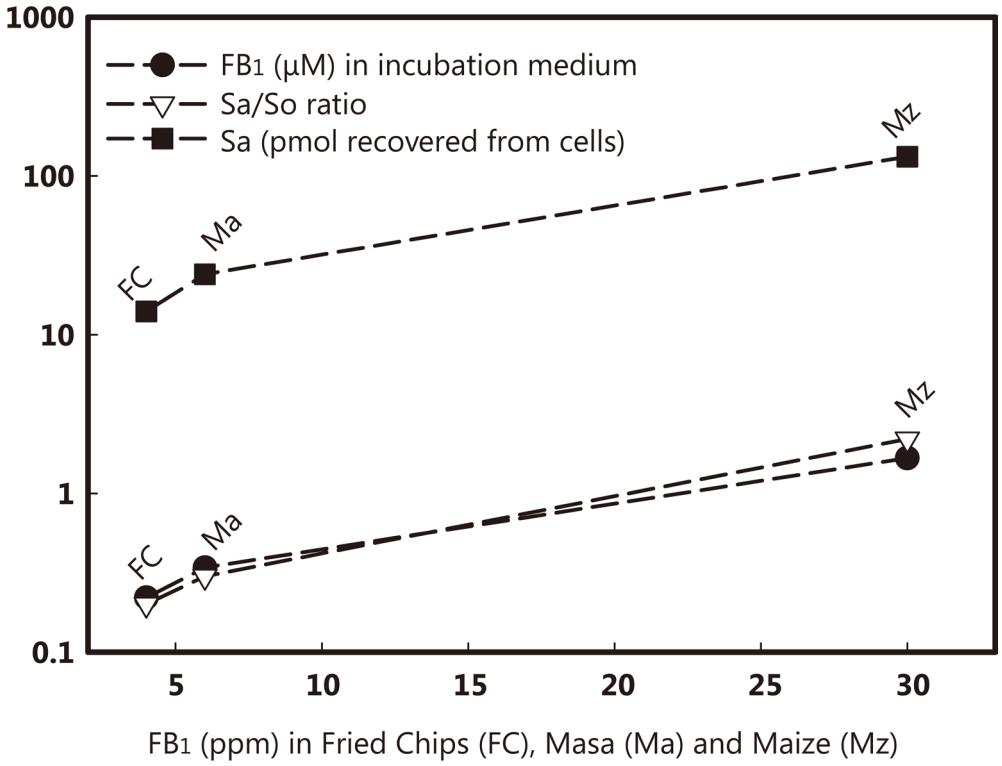

Fig. 4. Correlation between fumonisin B_1 (FB_1) concentration and biological activity of extracts of equivalent weights of maize (Mz), masa (Ma) and fried tortilla chips (Fc) made from the maize by *in vitro* bioassay using Vero cells, adapted from Voss *et al*. [59]. The x-axis indicates FB_1 concentration in Mz, Ma and Fc before extraction. Closed circles indicate FB_1 concentrations in the incubation media after the addition of the extracts (representing equivalent weights of Mz, Ma and Fc). Ceramide synthase inhibition was determined by measuring the amount of sphinganine (Sa; closed square) recovered and sphinganine:sphingosine ratio (Sa/So; open triangle) in the media after lysis of the cells (see text).

in masa and tortilla product extracts was significantly less than that of the uncooked maize. Reductions in activity were about 60% in the "Mayan tortilla" extracts[58]. Under larger-scale commercial conditions, the reductions were over 80% in masa and tortilla chips[59] (**Fig. 4**). While the results clearly indicated that fumonisin concentrations and biological (ceramide synthase inhibitory) activity were tightly correlated, they did not account for fumonisins or unknown reaction products that, due to poor extractability, were left behind in the maize, masa, or tortilla product.

In vivo (rat feeding) bioassays were therefore conducted[60]. First, the inherent potential of alkaline cooking to reduce toxicity and sphingolipid effects was tested using ground *F. verticillioides* culture material as a model. Rats were fed diets to which equivalent weights of unprocessed (positive control) or nixtamalized ground *F. verticillioides* culture material were added. Control groups were fed diets formulated with unprocessed (negative control) or nixtamalized (cooking control) uncontaminated, ground maize.

Unprocessed culture material caused significant apoptotic lesions and markedly increased sphinganine, sphinganine 1-phosphate and total sphinganine (sphinganine plus sphinganine 1-phosphate) concentrations in the kidneys after both one and three weeks of feeding. Significantly less severe effects were found in rats fed the nixtamalized culture material: apoptosis was reduced by 80–90% and total sphinganine, while still elevated compared to the negative and cooking control groups, was reduced by 30% (after one week) to 35% (after three weeks) compared to rats fed the unprocessed culture material.

Reduced toxicity was associated with the significantly lower FB_1 and increased HFB_1 concentrations in the culture material after nixtamalization: specifically, FB_1 levels were 9.1 ppm and 2.1 ppm and HFB_1 levels were 0.3 and 1.3 ppm in diets made from culture material and nixtamalized culture materials, respectively. FB_1 concentrations were further reduced (to 0.5 ppm in the diet), without significantly affecting HFB_1 (1.6 ppm) concentrations, by nixtamalization if the culture material was mixed with ground, uncontaminated maize before cooking. The culture material-maize mix diet did not elicit apoptotic effects or sphingolipid changes in the bioassay. Altogether, the findings showed (a) that nixtamalization significantly reduced *in vivo* toxicity and FB_1 concentrations; (b) that HFB_1 was less toxic than FB_1; and (c)

that FB_1 might under alkaline conditions possibly become irreversibly bound to or otherwise interact with maize matrix components in a manner that reduces its bioavailability, biological activity, or both. The supposition that matrix interaction might play a role is supported by semi-quantitative HPLC analyses (results not corrected for recovery) showing that a higher percentage (10 to 15%) of FB_1 in two batches of nixtamalized whole kernel maize was bound or otherwise tightly associated with the matrix, whereas less than 6% of the total FB_1 in the kernels before alkaline cooking was matrix associated[61].

In a second *in vivo* bioassay, the effect of nixtamalization on toxicity of FB_1 in three batches of whole kernel maize was tested[62]. One half of each batch was nixtamalized and then equivalent amounts (50% w/w in the formulated rations) of the uncooked and cooked portions were fed to male rats for three weeks. The diets made with uncooked maize (estimated FB_1 concentrations in the three formulated rations were 1.8, 3.6, and 4.2 ppm) caused mild to moderate apoptotic lesions in the kidneys and significantly elevated renal total sphinganine (ca. 480 to 623 nmol/g tissue). In contrast, no lesions or sphingolipid effects (total sphinganine 11 to 46 nmol/g tissue) were found in two of the groups fed nixtamalized maize. FB_1 concentrations in these diets were reduced by nixtamalization by more than 90%: from 1.8 to 0.08 ppm and from 3.6 to 0.13 ppm. Nixtamalization also reduced FB_1 concentration of the most highly contaminated maize batch by about 90% (from 4.2 to 0.37 ppm in the formulated rations) but only partially reversed toxicity as minimum to mild (as opposed to mild to moderate) apoptotic lesions were found in the kidneys. In this case, nixtamalization decreased total kidney sphinganine by about 50% (300 nmol/g tissue compared to 623 nmol/g in rats fed the corresponding uncooked maize) however; total sphinganine was 7 to 30 fold higher than in groups fed nixtamalized uncontaminated maize.

In summary, this series of analytical, *in vitro* and *in vivo* investigations have clearly shown that nixtamalization effectively reduces fumonisin toxicity through a combination of extracting the mycotoxin into the cooking-steeping-rinsing liquids, converting it to less toxic hydrolyzed species, and perhaps by promoting mycotoxin-matrix interactions that reduce its bioavailability. The results further showed that the benefit of alkaline cooking will be limited as fumonisin levels in maize increase to some critical, and as yet undetermined, level.

Reproduction and Development

FB_1 was overtly or indirectly (secondary to maternal toxicity) fetotoxic when orally administered to pregnant Syrian hamsters[63], rabbits[64], mice[65] and rats[66]. Growth retardation, delayed or incomplete ossifications, and occasional variations or malformations including low incidences of cleft palate or hydrocephalus, and fetal death were among the findings in one or more species. Effects were generally dose-dependent and, based on histopathological and sphingolipid profile assessments of the dams, considered secondary to maternal toxicity.

FB_1 and Neural Tube Defects (NTD)

NTD are birth defects of the brain and spinal cord resulting from failure of the neural tube to properly close early in gestation; in humans, during the first month of pregnancy. Clinical presentation is variable and includes spinal bifida, anencephaly, meningio-myeloceole or craniorhachischisis (externalization of brain and spinal cord). The etiology is not well understood, complex and involves genetic, environmental and nutritional factors. The latter includes folate[12], an essential nutrient that is critical for one-carbon transfer reactions, e.g. methylation of DNA. Clinical and epidemiological studies have shown that supplementation during early pregnancy reduces NTD risk[12]. It is of concern that maize is naturally low in folate and that nixtamalization further reduces it levels, thereby possibly increasing NTD risk[12].

In areas of southern Africa, China, Mexico and Guatemala where maize constitutes the major part of the diet, the annual NTD rate is six or more times higher than the average worldwide rate[12,13,67]. In the United States, there was an NTD cluster in 1990–1991 in which an almost 2-fold increase in its incidence, from 15/10,000 to 27/10,000, was noted among babies born to Mexican-American women in southeastern Texas[13]. Relatively high fumonisin concentrations in locally grown maize together with a concurrent increase in equine leukoencephalomalacia cases in the area suggested a connection between fumonisins and NTD.

Epidemiology and *in Vitro* Studies

Results of a retrospective epidemiological study of the Texas NTD cluster showed a correlation between NTD and moderate tortilla consumption (300 to 400) during the first trimester of pregnancy[13]. NTD rates fell off again as tortilla

consumption increased beyond 400, perhaps as a result of increased fetal death. Estimated FB_1 exposures together with sphingolipid analysis results suggested a similar "inverted U" dose-response between NTD risk and exposure.

The first experimental evidence that FB_1 might increase NTD risk was the finding that FB_1 inhibited 5-tetramethylhydrofolate uptake and utilization in cultured CaCo2 cells[68]. Inhibition was correlated with FB_1-dependent reduction of critical complex sphingolipids associated with the high affinity GPI-anchored folate receptor (designated Folr1 in mice; FRα in humans) which is located in sphingolipid and cholesterol rich membrane rafts[12,68]. FB_1 also retarded growth and induced NTD in cultured mouse embryos; however, the addition of folinic acid to the culture medium reduced the number of embryos developing NTD, thus partially reversing the effect of FB_1[69].

In Vivo Experiments

The teratogenic potential of FB_1 in mice was first evaluated using the CD-1 strain[65]. Fetal effects (hydrocephalus) occurred at maternal doses of ≥25 mg/kg body weight (intragastric gavage) on embryonic days 7 through 15 (E7-E15) and fetal death rates were increased at 100 mg/kg body weight, the highest dose studied. Fetal findings did not include NTD and were judged secondary to maternal toxicity (hepatic lesions and sphingolipid metabolism disruption) caused by FB_1 at doses ≥25 mg/kg body weight.

NTD (exencephaly) were induced however when FB_1 was given to inbred LM/Bc mice by intraperitoneal injection (ip) at doses of 0, 5, 10, 15 and 20 mg/kg body weight (n =10/group) on E7.5 and E8.5[70], which is the critical "window" for neural tube closure during development. The effect was dose-dependent: the incidences of dams having at least one NTD-affected fetus were 0, 40 and 70% at doses of 0, 5 and 10 mg/kg body weight respectively and 100% at higher (≥15 mg/kg body weight) doses. The percentage of NTD affected fetuses/group also increased dose-dependently from 5% at 5 mg/kg body weight to 79% at the high dose of 20 mg/kg body weight.

Consistent with the *in vitro* studies[68,69], co-exposure to folic acid (20 mg/kg body weight given ip daily on E0.5 through E9.5) and FB_1 (20 mg/kg body weight on E7.5 and E8.5) lowered the NTD rate to 50%. The complex sphingolipid GM_1 (10 mg/kg ip on E6.5–9.5) provided greater protection as only 5% of fetuses from dams co-exposed to GM_1 and the aforementioned dose of FB_1 were exencephalic. Confocal microscopy and immunohistochemistry revealed the co-localization of Folr1 and GM_1 in the yolk sac membrane and, moreover, that staining of the sphingolipid associated GPI-anchored Folr1 was markedly diminished by FB_1. In contrast, SWV mice are resistant to NTD induction by FB_1[12], possibly because of the lesser degree of sphingolipid metabolism disruption occurring in response to exposure.

Although FB_1 did not induce NTD when orally (gavage) given to CD-1 dams at 12.5 to 100 mg/kg body weight during organogenesis[65], it did cause exencephaly in CD-1 fetuses when given ip to the dams during the E7 and E8 (corresponds to E7.5 and E8.5 in Gelineau-van Waes *et al*. [70]) "window" for neural tube closure[71]. The dose-response was "shifted to the right" when compared to LM/Bc mice: the percentage of CD-1 dams having one or more fetuses with NTD was 0, 8, 11, 17, 0, 38 and 45 at doses of 0, 10, 15, 23, 30, 45 and 100 mg/kg body weight. NTD rates in the positive litters increased dose-dependently from 8% at the low dose to 41% at the high dose of 100 mg/kg body weight. Some evidence suggests that sphingolipid metabolism of CD-1 mice, like that of SWV mice, is less extensively disrupted by FB_1 than in the LM/Bc strain[72–74]. Differences in sensitivity among the three strains can be exploited experimentally for elucidating the mechanisms and genetic factors underlying NTD induction.

In any event, the relevance of the findings in mice to humans exposed to fumonisin via the diet is unclear. First, the pharmacokinetics following ip injection has not been studied, but is surely quite different from that encountered in dietary exposure. Furthermore, assuming that gastrointestinal absorption of FB_1 in mice is in the three to 5% range as has been reported for rats[5,6], an ip dose of 5 mg/kg body weight corresponds to approximately 200 to 300 ppm FB_1 in the feed. These values are about six to 11 fold higher, respectively, than the NOEL of 27 ppm that was established for female mice in a 13-week feeding study[24]. However, it should be noted that NTD were found in 20% of LM/Bc fetuses following exposure of the dams to 20 mg/kg body weight FB_1 given by oral gavage[70]. Further defining dose-response to find the NOEL and LOEL for NTD induction in LM/Bc mouse following oral exposure to FB_1 is a focus on ongoing experiments.

Other Mechanistic Aspects Affecting Reproduction

The sphingoid base 1-phosphates are ligands for a family of G protein couple receptors residing in cell membranes known as sphingosine 1-phosphate receptors, or S1PR[74]. Sphingosine 1-phosphate likely plays a critical role in neural tube closure and other aspects of mouse embryo development[12,74] so it can be supposed that inappropriate S1PR signaling resulting from elevated sphingoid base 1-phosphate levels could at least in part contribute to NTD induction by FB_1.

FTY720 (Fingolimod) is a synthetic sphingoid base analog. Like sphinganine, it is phosphorylated by a sphingosine kinase (sphingosine kinase 2) to its 1-phosphate metabolite (FTY-1P) which is an agonist for at least three members of the S1PR family. When LM/Bc dams (n =10) were given daily oral doses (E6.5–8.5) of 10 mg/kg body weight FTY720, 62% of the embryos exhibited NTD[74]. Similar treatment of SWV dams resulted in a lower, 40%, NTD incidence. All litters of both strains had at least one fetus exhibiting failed neural tube closure. Both FTY720 and FTY-1P (structurally similar to sphingosine 1-phosphate) accumulated in maternal blood and plasma of both strains with higher amounts of each being found in LM/Bc dams. FTY720 and perhaps FTY-1P (phosphorylation in embryo tissues could not be discounted) crossed the placenta as both were detected in exancephalic embryos of both strains. Their concentrations were however significantly higher in LM/Bc embryos. These results act as "proof of concept" providing evidence that inappropriate antagonism of S1PR via sphingoid base 1-phosphates has a mechanistic role in the induction of NTD in mice.

Co-exposure to tetrahydrobiopterin (BH_4), like GM_1 treatment, reduced NTD rates in LM/Bc fetuses by about 95% when tested according to the protocol of Gelineau-van Waes *et al.* [73]. The first step of BH_4 biosynthesis is mediated by the enzyme GTP cyclohydrolase (GTPCH) and both BH_4 production and GTPCH are detectable during the early stages of development in neural crest cells. The manner by which BH_4 was protective is not fully clear but it can be speculated that it involves disruption of nitric oxide (NO) metabolism[73]. Briefly, BH_4 is one of several cofactors for the nitric oxide synthase (NOS) mediated production of NO (plus citrulline) from molecular oxygen (plus L-arginine). If cellular BH_4 levels are depleted, the reaction becomes "uncoupled", resulting in production of reactive oxygen species such as superoxide and hydrogen peroxide instead of citrulline and NO. Since NO contributes to control of cell cycle progression and maintaining the proper balance of apoptosis and cell proliferation during neural tube closure, its depletion can adversely affect development. In addition, oxidative stress caused by the reactive oxygen species generated by the "uncoupled" enzyme can also contribute.

Feeding Studies with *F. verticillioides* Culture Material

Attempts to induce NTD in mice by feeding FB_1-contaminated diets have been few and inconclusive. LM/Bc and CD-1 mice were fed diets nominally containing 50 or 150 ppm FB_1 (contributed by the addition of *F. verticillioides* culture material to the ration) beginning five weeks before they were paired with naïve males[72]. Dietary exposure continued during mating and gestation. A control group was fed uncontaminated maize. Estimated daily FB_1 intakes in the low-level groups during the five-weeks before mating were 8 and 13 mg/kg body weight respectively for the LM/Bc and CD-1 dams. Intakes averaged 25 and 39 mg/kg body weight for the respective strains fed the high-level diets.

Examination of the dams and fetuses on E16 did not reveal any differences in the number of implants, resorptions, late fetal deaths or live fetuses. A single fetus in one of five high-dose LM/Bc litters did however exhibit exencephaly. Otherwise, there were no remarkable external abnormalities. Maternal toxicity, i.e. hepatic apoptotic lesions and altered sphingolipid profiles, was evident in both strains, but only at the high-dose. A slight but dose-related increase (significant at the high-dose, $P < 0.05$) in liver sphinganine and sphinganine 1-phosphate concentrations of LM/Bc fetuses was found. In contrast, liver sphinganine and sphinganine 1-phosphate concentrations of the CD-1 fetuses did not differ from those of control litters.

No NTD were found however when the study was repeated, this time feeding the LM/Bc females diets formulated with the culture material to contain nominal concentrations of 150 or 300 ppm FB_1[73]. Fetal toxicity was evident in this case as a dose-related increase in resorptions that was significant ($P < 0.05$) in the high-dose group. The absence of NTD in the second experiment could be due to one or more of the following in combination: (a) the unlikely event that the NTD in the first experiment was spontaneous; (b) physiological adaptation of the dams to fumonisins during the extended exposure period, for example up- or down-regulation of critical genes; (c) pharmacokinetic considerations resulting in lower or slower absorption and lower peak serum levels of FB_1 during dietary exposure; (d) FB_1-diet interactions reducing bioavailability; and (e) the presence of constituents in the culture material that protect against NTD induction. In regard to the latter possibility, the folate content of *F. verticillioides* culture material has yet to be defined and experiments are underway to explore how folate levels in the diet affect FB_1-dependent NTD induction. It is of interest that preliminary findings suggest that feeding folate deficient diet to LM/Bc females beginning five weeks prior to mating significantly reduces maternal red blood cell folate concentrations while not exacerbating FB_1-dependent NTD (unpublished data).

HFB_1 and NTD

Varying concentrations of HFB_1 occur in nixtamalized foods[5,6] so that the likelihood of exposure during pregnancy is especially high in populations where maize-based, alkaline cooked foods are a major component of the diet. NTD and other developmental abnormalities were induced in rat embryos *ex utero* when HFB_1 concentrations in the incubation media were ≥100 μmol[75], suggesting that HFB_1 might be a risk factor for NTD.

To determine the teratogenic potential of HFB_1 *in vivo*, ip doses of 0, 2.5, 5.0, 10 or 20 mg/kg body weight HFB_1, which correspond to 6, 12, 25 and 50 μmol/kg body weight, were given to LM/Bc dams during the two-day "window" for neural tube closure[76]. Negative and positive control groups were given vehicle or 10 mg/kg (14 μmol/kg) body weight FB_1, respectively. One-half of the dams were killed on E9 for evaluation of maternal toxicity including liver histopathology and tissue sphingolipid concentrations. The remaining dams and litters were examined on E16.

FB_1 significantly reduced the number of viable fetuses per litter, decreased fetal and placental weights, and induced NTD in all litters: NTD were found in two-thirds of the fetuses. In contrast, HFB_1 neither induced NTD nor had any adverse effect on the reproductive variables evaluated including early (resorptions) and late fetal death counts, live fetus counts, live fetal weight, or placental weight.

FB_1 clearly caused maternal toxicity as evidenced by moderate hepatic liver lesions and marked increases in hepatic sphinganine, sphingosine, and their 1-phosphates at E9. 1-Deoxysphinganine and 1-deoxysphingosine concentrations were also significantly ($P < 0.05$) elevated at E9, whereas complex sphingolipid concentrations were significantly decreased.

HFB_1 did not cause liver lesions but did affect maternal sphinganine concentrations in a dose-dependent manner at ≥5 mg/kg body weight. Elevations were however minimal compared to the extensive increases in dams treated with FB_1 and, compared to vehicle controls, a statistical significance ($P < 0.05$) was demonstrated only at the high-dose. Concentrations of sphingosine, sphinganine 1-phosphate and sphingosine 1-phosphate were also increased and complex sphingolipids were decreased at 20 mg/kg body weight HFB_1; but again, these effects were much less pronounced than in dams given 10 mg/kg FB_1 body weight. The finding that complex sphingolipids in placenta of dams in the FB_1-treated positive control group were decreased at E9 is consistent with the concept that depletion of complex sphingolipids associated with the folate receptor is a key event modulating folate utilization in the LM/Bc mouse model for NTD induction by FB_1.

Placentas from the vehicle, FB_1 and high-dose HFB_1 treated groups were also evaluated for sphingolipid effects on E16. No differences between the latter group and the vehicle controls were found. In contrast, sphinganine, deoxysphinganine, and deoxysphingosine were significantly increased and sphinganine 1-phosphate and sphingosine 1-phosphate concentrations decreased in the FB_1 treated group. No significant differences in complex sphingolipid concentrations were found although they tended to be lower in the dams given FB_1 than in dams from the control group.

The findings demonstrated that HFB_1 is not a significant risk factor for NTD. They further showed that HFB_1 caused only slight disruption of maternal sphingolipid metabolism at doses up to seven-fold higher than a dose of FB_1 that severely affected sphingolipid homeostasis. The results also complement the findings of the 28-day feeding study of Howard *et al*.[33] who found no evidence of toxicity in female mice fed HFB_1 whereas diets containing an equivalent amount (molar basis) of FB_1 were clearly hepatotoxic.

Biomarkers

The association between fumonisin exposure and esophageal cancer[5,6], neural tube defects[12,13,67], stunting[77] or other effects in humans[5,6,11] are based on correlations drawn from observations "in the field" (i.e. comparison of FB_1 in maize from areas having high or low incidences of esophageal cancer), findings from laboratory and farm animal experiments, or a combination of both. Carefully designed epidemiological investigations are needed to establish the extent that fumonisins affect human health. This in turn depends upon the availability of reliable, robust biomarkers of exposure.

Sphinganine and Sa/So

The tight correlations between exposure, increased sphinganine concentration or Sa/So in tissues, serum and urine (as reviewed in the "Mechanism" and "Characterization of Liver and Kidney Toxicity" sections), and organ-specific effects in animals was recognized early on by various research groups as a potential biomarker for epidemiological

purposes. While the approach has been useful for controlled laboratory experiments, its use in humans has been problematic: some investigators suggested a correlation between estimated exposures and urinary Sa/So[78] whereas others have found no relationship[79–81].

One confounding factor to the use of sphinganine concentration or Sa/So is the rapid reversibility of fumonisin-dependent sphingolipid effects as has been shown to occur after acute exposure or following replacement of contaminated feed with a "sound" diet[37,39,40,76]. Therefore, failure to find elevated serum or urinary sphinganine in a study cohort does not preclude the possibility that significant fumonisin exposure had occurred at an earlier and perhaps critical time. Another consideration is the possibility that an extended low-level exposure that follows an acute high-level exposure might maintain tissue or serum sphinganine concentrations at levels that are higher than would be encountered at the same low exposure level in the absence of previous acute exposure. This phenomenon was found to occur in rats[32] and mice[82] and should be further investigated. Validation of a biomarker also requires confident quantification of fumonisins in the dietary sources of exposure for purposes of estimating daily mycotoxin intake. Adequate analytical methods are available for quantifying FB_1 and other congeners in food. However, heterogeneity of mycotoxin distribution in commodities and foods as well as uncertainties related to the timing and method (to assure the sample is representative) of sampling confound the validation and use of sphingolipid (and other) biomarkers of exposure.

Urinary FB_1 Excretion

A second strategy is the use of urinary FB_1 excretion as an indicator of exposure, as suggested by the findings of Cai *et al.* [83] who demonstrated a dose-response related increase in urinary FB_1 in rats that had been orally dosed with up to 2.5 mg/kg body weight per day for five weeks. Several research groups have now applied this approach to human epidemiological and intervention studies.

In an investigation conducted in Mexico, Gong *et al.* [84] did indeed find significant correlation between maize tortilla consumption, which served as a surrogate for fumonisin exposure, and urinary FB_1 excretion. In a another study, urinary FB_1 was significantly higher in maize consuming adults from Huaian, China (estimated FB_1 intake 460 μg/kg body weight per day) compared to those living in Fusui (estimated FB_1 intake 127 μg/kg body weight per day)[81]. The urinary FB_1 biomarker has also been used to support the effectiveness of kernel sorting and washing[85] or dietary administration of a montmorillonite clay sequestering agent[86] as potential interventions to reduce fumonisin intakes in Africa.

Excretion Kinetics

Volunteers (n =8) were enrolled to study the kinetics of FB_1 excretion in urine following consumption of 206 g/day of bisquits (1.3 ppm total FB_1 + FB_2 +FB_3; HFB_1<LOD of 0.01 ppm) and tortillas (total FB_1 + FB_2 +FB_3;=2.2 ppm; HFB_1=0.1 ppm) made from commercially purchased, maize-based hot cereal/atol or masa flour, respectively[87]. After abstaining from maize foods for three days, they ate the bisquits and tortillas for three consecutive days, and abstained again for another five days. Urine samples were collected for LC-MS quantification of fumonisins and total excretion per day was recorded during the consumption and post consumption periods.

Despite the presence of the three fumonisins in the foods at average ratios of 1.0:0.30:0.15, no FB_2 or FB_3, (LOD =0.04–0.07 ng/ml) could be detected in the urine. This finding is similar to observations in rats[36] and primates[88,89] and suggests that gastrointestinal absorption of FB_1 is significantly higher than that of the other congeners. More efficient excretion of FB_1 is an alternative explanation if it is assumed that absorption of FB_1, FB_2, and FB_3 occurs with approximately equal efficiency. HFB_1 (LOD =0.18 ng/ml) was also not detected in the urine. The average daily FB_1 intake for the eight volunteers was 2.94 μg/kg body weight.

FB_1 was detected in urine of all volunteers on the first day of consumption, remained relatively constant for the final two days of consumption, declined rapidly after exposure was stopped, and was no longer detectable on the third day post exposure. Excretion rates among individuals were highly variable. On average, 10 to 20 ng FB_1/kg body weight was excreted per day, a value that corresponded to a total excretion of about 1 to 2 μg FB_1 per person each day and about 0.5% of the total FB_1 consumed. Similar results were found in two small scale preliminary trials[87].

FB_1 was also detected in the urine (mean value =0.3 ng/ml) of 136 of 177 healthy consumers of maize foods (≥410 g/day, predominantly tortillas) recruited from two departamentos in Guatemala[87]. Assuming that the concentrations of FB_1 found in purchased maize (mean value =0.36 ppm) accurately reflected levels in maize used for masa production as well as making assumptions for the effect of nixtamalization (50% reduction in FB_1 in tortillas and other nixtamalized foods relative to maize) and for urinary output (1000 mL/day), the daily intake of FB_1 was estimated to be about 0.45 μg/kg body weight, well below the JECFA PMTDI. The estimated average FB_1 intake was 30 μg total for a person of average

weight (67 kg) and urinary excretion was on average 0.3 µg fumonisin per day, which was about 1% of the total estimated fumonisin intake. Ongoing studies in Guatemala are focused on determining if a relationship can be established between urinary FB_1 excretion (biomarker of exposure) and an elevated sphinganine 1-phosphate:sphingosine 1-phosphate ratio in red blood cells (mechanistic biomarker of effect) of consenting volunteers[90].

Conclusion

The basic and applied toxicological and other studies highlighted in this review represent only a portion of the multidisciplinary initiatives by research groups from around the world that have been undertaken since then and have contributed to the establishment of 2 µg/kg body weight PMTDI for fumonisins B_1, B_2 and B_3 alone or in combination[5,6]. However, despite significant progress on many fronts, it is still not known to what extent or under what circumstances FB_1 or other fumonisins might be a risk factor for cancer, birth defects or other diseases in humans.

Development and validation of robust, fumonisin-specific biomarkers to better assess exposures and to establish whether or not fumonisins disrupt sphingolipid metabolism in humans is a high priority. Results of studies in rats[14] and rainbow trout[15] have demonstrated enhanced promotion of aflatoxin B_1 carcinogenesis by fumonisin B_1, thereby pointing to the critical need for further investigations on the consequences of co-exposure. A recent study in Tanzanian children has shown that co-exposure to high levels of both mycotoxins is common[91]. Another priority is to further exploit the species- and strain-related differences in target organs and sensitivity to fumonisins in comparative, mechanistic and genomic/epigenomic-based experiments to determine which species/strains and toxicological outcomes provide the most relevant models for risk assessment. In conclusion, while much has been learned about fumonisins and their toxicology, significant questions remain that must be investigated to better understand the impact of these widespread mycotoxins on human health.

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