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Research Article

Hydrolyzed fumonisins HFB_1 and HFB_2 are acylated *in vitro* and *in vivo* by ceramide synthase to form cytotoxic *N*-acyl-metabolites

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Fumonisins B_1 and B_2 (FB_1 and FB_2) are the most abundant members of the fumonisins - mycotoxins that are produced by *Fusarium verticillioides* and are natural inhibitors of ceramide synthase. Their hydrolyzed forms, HFB_1 and HFB_2 (also called AP_1 and AP_2) are found in some foods, and they are not only inhibitors of ceramide synthase but also undergo acylation by this enzyme. This study characterized the conversion of HFB_1 and HFB_2 by ceramide synthase to their respective *N*-acylated metabolites using rat liver microsomes and palmitoyl-CoA or nervonoyl-CoA as cosubstrates, and examined animals that had been dosed with hydrolyzed fumonisins to ascertain if acylation occurs *in vivo*. Using an HPLC-MS/MS method that allowed the sensitive and selective detection of the acylation products, both HFB_1 and HFB_2 were found to be metabolized *in vitro* to nervonoyl- or palmitoyl-HFB_1 and -HFB_2 (*i.e.* $C_{24:1}$-$HFB_{1/2}$ and C_{16}-$HFB_{1/2}$, respectively). The apparent v_{max} was considerably higher for formation of $C_{24:1}HFB_1$ (157 pmol/min/mg protein) than for formation of $C_{16}HFB_1$ (8.7 pmol/min/mg protein). The acylation products also inhibited ceramide synthase and significantly reduced the number of viable cells in an *in vitro* [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)] assay using a human colonic cell line (HT29). Furthermore, HPLC-MS/MS analysis of tissues from rats given intraperitoneal doses of HFB_1 confirmed that formation of *N*-acyl-HFB_1 occurs *in vivo* to produce metabolites with fatty acids of various chain lengths. The contribution of acylated HFB_1 and HFB_2 metabolites to fumonisin toxicity *in vivo* warrants further investigation.

Keywords: Ceramide synthase / Cytotoxic / HPLC-MS / Hydrolyzed fumonisins / *N*-Acyl-metabolites

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1 Introduction

Fumonisins are mycotoxins produced by *Fusarium verticillioides* (the most prevalent fungus associated with corn worldwide) and are common contaminants of corn and corn products [1]. Structurally, fumonisins are comprised of a long-chain backbone with two of the hydroxyls esterified to tricarballylic acid (TCA). Of the more than 28 fumonisin isomers known [2], fumonisin B_1 (FB_1), B_2 (FB_2) and B_3 (FB_3) are the most abundant (Fig. 1). Fumonisins that lack the TCA side chains are referred to as hydrolyzed fumonisins ($HFB_{1/2}$) or aminopolyols ($AP_{1/2}$) (Fig. 1) and are formed during the production of masa (tortilla flour) by base treatment (nixtamalization) [3, 4].

Consumption of *F. verticillioides*-contaminated corn by horses and swine causes equine leukoencephalomalacia [5,

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Abbreviations: C_n-$HFB_{1/2}$, *N*-acylated hydrolyzed fumonisins B_1 and/or B_2 with fatty acids of various chain length (n = 16, 18, 20, 22, 24, 24:1); **CoA**, coenzyme A; **EDC**, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; **$FB_{1/2}$**, fumonisin B_1 and/or B_2; **$HFB_{1/2}$**, hydrolyzed fumonisin B_1 and/or B_2; **MRM**, multiple reaction monitoring

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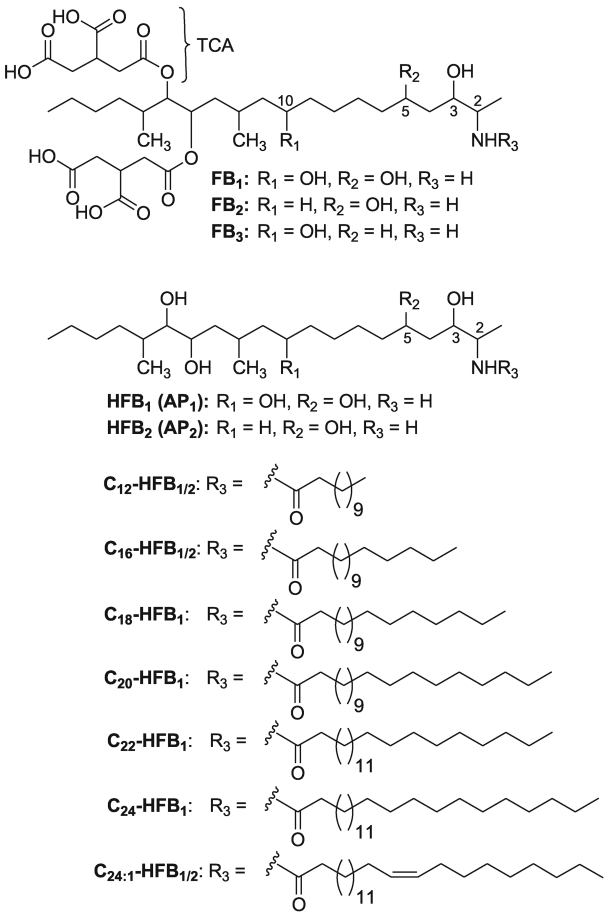

Figure 1. Structures of fumonisins FB_1, FB_2 and FB_3, hydrolyzed fumonisins (aminopolyols) HFB_1 and HFB_2 and the corresponding *N*-acyl-derivatives.

6] and porcine pulmonary edema [7, 8], respectively. FB_1 is hepatotoxic and nephrotoxic to a variety of species and is a liver and kidney carcinogen in rodents [1, 9 – 13]. Concerning their impact on humans, fumonisins have been implicated as a possible cause of esophageal cancer in China and South Africa [14, 15] and it has furthermore been hypothesized that fumonisins are a risk factor for neural tube defects (NTD) [16], for which there has been recent epidemiologic evidence [17]. The likely explanation for the ability of fumonisins to have such diverse toxicities is that their mechanism of action is to inhibit ceramide synthase(s), the enzymes catalyzing the acylation of sphinganine and sphingosine to dihydroceramides and ceramides, respectively [18 – 21]. This disruption of sphingolipid metabolism causes a decrease of complex sphingolipids and an accumulation of highly bioactive sphingoid bases, sphinganine and sphingosine, and their 1-phosphate metabolites [18 – 22]. The toxicological implications are reviewed elsewhere [18 – 22].

The hydrolyzed form of FB_1 has also been shown to be an inhibitor of ceramide synthase, but HFB_1 is tenfold less potent compared to FB_1 and it is cytotoxic, although less toxic than FB_1 in cell culture [23]. There is conflicting evidence concerning the *in vivo* toxicity of hydrolyzed fumonisins. Hepatic and renal lesions found in rats fed hydrolyzed *F. verticillioides* culture materials containing HFB_1 (but no detectable FB_1) were qualitatively similar to those caused by FB_1 [24, 25] whereas purified HFB_1 did not cause liver or kidney apoptosis or disrupt sphingolipid metabolism when fed to female mice [26] or given by gavage to pregnant rats [27]. The reasons underlying these apparently contradictory results have not been established. In studies of the possible mechanisms whereby hydrolyzed fumonisins are toxic, HFB_1 was found to be acylated by ceramide synthase to form the metabolite *N*-palmitoyl-aminopolyol (C_{16}-HFB_1), which was toxic to cells in culture [28]. Since that publication, it has been found that there are multiple isoforms of ceramide synthases (CerS) that differ in fatty acyl-coenzyme A (CoA) selectivity [29 – 32]. The aims of this study were to further characterize the acylation of HFB_1 and HFB_2 *in vitro*, to study the biological activities of long-chain and very long-chain *N*-acylated hydrolyzed fumonisin metabolites *in vitro*, and to demonstrate that *N*-acylation of HFB_1 occurs *in vivo*.

2 Materials and methods

2.1 Materials

FB_1, FB_2 and HFB_1 were supplied by Dr. M. Trucksess from the US Food and Drug Administration, Washington, DC, USA. HFB_2 was produced from FB_2 according to the method of Hopmans *et al*. [33] and further purified by a C18 cartridge. HFB_1 for the animal experiment was prepared from semipurified FB_1 extracted from *F. verticillioides* culture material (approximately 75% purity; a gift from Filmore Meredith, Toxicology & Mycotoxin Research Unit, Agricultural Research Service-USDA, Athens, GA). Briefly, the FB_1 was dissolved in 2 N KOH and quantitatively transferred (two washes with 2 N KOH) to a Teflon-capped test tube. The tube was shaken, sonicated briefly (< 1 min) and heated (70°C) for approximately 20 h. After cooling to ambient temperature, the pH was adjusted to 4.0 using dilute HCl and then brought to volume (110 mL) with deionized water. For cleanup, the HFB_1 solution was loaded onto a preconditioned C18 cartridge. The cartridge was washed with 100 mL water and 100 mL 15% aqueous ACN, after which the HFB_1 was eluted with 100 mL of 70% ACN. Purity (> 95%) of the HFB_1 was assessed by comparison of the HFB_1 to an analytical standard by HPLC-MS (SIM) (Stephen Poling, ARS-USDA, Peoria, IL).

Silica gel and C18 SPE columns were purchased from Waters (Milford, MA). D-*erythro*-[3-^{3}H]sphingosine was from NEN Life science Products. All other chemicals were of reagent grade.

2.2 Analytical methods

HPLC-ESI-MS/MS analysis of ceramide synthase acylation products were performed on a Perkin-Elmer Sciex API 3000 triple stage quadrupole mass spectrometer equipped with a turboionspray source. Product and precursor ion analyses involved infusion of synthetic C_{12}-$HFB_{1/2}$, C_{16}-$HFB_{1/2}$, and $C_{24:1}$-$HFB_{1/2}$ at a flow rate of 5 μL/min. These solutions were comprised of 5 mM ammonium acetate in MeOH and 1% acetic acid. The synthetic HFB_1 species were present at ~100 fmol/μL. In multiple reaction monitoring (MRM) analyses, effluent from the HPLC entered the turboionspray source at a flow rate of 400 μL/min. Dry N_2 was used as a nebulizing gas at a flow rate of 6 L/min and the face plate was heated to 500°C. In all analyses, the ion spray needle was held at 5500 V while the orifice and skimmer voltages were kept low to minimize collisional activation of molecular ions prior to entry into the first quadrupole. Product ion spectra were acquired by setting Q1 to pass the protonated molecular ion of interest. These were *m/z* 406.5, 588.7, 644.7, and 754.8 for HFB_1, C_{12}-HFB_1, C_{16}-HFB_1, and $C_{24:1}$-HFB_1, respectively, as well as *m/z* 390.4, 572.7, 628.7, 740.8 for HFB_2, C_{12}-HFB_2, C_{16}-HFB_2, and $C_{24:1}$-HFB_2, respectively. The $[M+H]^+$ ions were induced to dissociate by collision with N_2 in Q2 which was offset from Q1 by 35 eV. Subsequently formed product ions were detected by scanning Q3 in 0.3-u steps from *m/z* 50–800 with a dwell time of 1 ms. Precursor ion spectra were acquired by scanning Q1 over a 50-u window around each synthetic $HFB_{1/2}$ species estimated molecular mass in 0.1-u steps with a dwell time of 4.0 ms. Q2 was offset from Q1 by 35–65 eV to determine each species optimal collision energy with regard to formation of molecularly distinctive product ions. Therefore, Q3 was then set to pass product ions of *m/z* 334.4 and 336.3 for HFB_1 and HFB_2 species, respectively. Data was acquired for a total of 1 min, thus each spectrum is the signal averaged sum of 30 scans. MRM spectra were acquired by setting Q1 and Q3 to pass the precursor and product ion, respectively, which are specific to each individual species. The transitions monitored for HFB_1, and its *N*-acyl derivatives were *m/z* 406.5/334.4 (HFB_1), 588.7/334.4 (C_{12}-HFB_1), 644.7/334.4 (C_{16}-HFB_1), 672.7/334.4 (C_{18}-HFB_1), 700.7/334.4 (C_{20}-HFB_1), 728.7/334.4 (C_{22}-HFB_1), 754.8/334.4 ($C_{24:1}$-HFB_1), and 756.8/334.4 (C_{24}-HFB_1). Q2 was offset from Q1 by 35, 45, 47.5, 47.5, 50, 52.5, 55, and 55 eV to yield optimal signal intensity for each HFB_1 species. The transitions monitored for HFB_2, C_{12}-HFB_2, C_{16}-HFB_2, and $C_{24:1}$-HFB_2 were *m/z* 390.4/336.3, 572.7/336.6, 628.7/336.3, 738.8/336.3, respectively. Q2 was offset from Q1 by 30, 35, 40, and 50 eV to yield optimal signal for each HFB_2 species. The individual transitions were monitored with a dwell time of 25 ms each.

All HPLC analyses were performed using a Perkin-Elmer series 200 micropump and autosampler. Solvent A consisted of 69:30:1 v/v/v H_2O/CH_3OH/acetic acid, solvent B was 99:1 MeOH/acetic acid, and both contained 5 mM ammonium acetate. Samples were dissolved in 200 μL solvent A, and a total of 50 μL was injected onto a 15 cm × 2.1 mm Supelco Discovery C18 RP column. The $HFB_{1/2}$ series were eluted at a flow rate of 400 μL/min by pre-equilibration of the column by washing with 100% A for 2.5 min, followed by sample injection, 2-min wash with 100% A, a 5-min linear gradient to 100% B, an 8-min wash with 100% B, and a 2.5-min post-equilibration with 100% A.

The ^1H NMR spectra were recorded in $CDCl_3$ on a Bruker WM 400-MHz spectrometer and are reported in parts per billions relative to $CHCl_3$ (7.26 ppm) as an internal reference.

2.3 Synthesis of *N*-lauroyl, *N*-palmitoyl- and *N*-nervonoyl-HFB_1

To a stirred solution of HFB_1 (3 mg, 7.4 μmol), 1-hydroxybenzotriazole (HOBT; 1.1 mg, 8.2 μmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC; 1.6 mg, 8.4 μmol) and 8.2 μmol fatty acid (1.6 mg lauric acid, 2.1 mg palmitic acid or 3.0 mg nervonic acid) in 0.5 mL DMF was slowly added *N,N*-diisopropylethylamine (2.9 mg, 22.2 μmol). The reaction was allowed to stir overnight at room temperature under nitrogen atmosphere and product formation was checked by TLC using silica plates (Merck) and $CHCl_3$:MeOH (90:10, v/v). The fumonisins were visualized by dipping the plates into a 0.5% solution of *p*-anisaldehyde in MeOH:AcOH:H_2SO_4 (85:10:5, v/v/v) and heating them to 100°C. The reaction mixture was diluted with $CHCl_3$ to 4 mL, washed with 3 × 2 mL of each H_2O, brine and $NaHCO_3$. The $CHCl_3$ layer was dried over Na_2SO_4, concentrated and applied to a SiO_2 SPE cartridge (500 mg capacity; Waters) previously conditioned with 10 mL $CHCl_3$:MeOH (90:10, v/v) and 10 mL $CHCl_3$. The column was washed with 4 mL of $CHCl_3$, 4 mL of $CHCl_3$:MeOH (95:5, v/v) and eluted with 6 mL of $CHCl_3$:MeOH (90:10, v/v) in 0.5-mL steps. The fractions that contained the desired product were identified by TLC and then combined and the solvent was removed. The *N*-acylated products were characterized by ESI-MS/MS and ^1H NMR.

C_{12}-HFB_1: yield: 3.38 mg (78%)

^1H NMR (400 MHz, $CDCl_3$, ppm, *J* in Hz):

δ = 0.82 (t, *J* = 6.4, 3H), 0.87 (t, *J* = 7.0, 3H), 0.89 (t, *J* = 7.4, 3H), 0.98 (d, *J* = 7.0, 3H), 1.0–1.8 (m, 38H), 1.25 (brs, 3H), 1.94 (m, 2H), 2.34 (m, 1H), 2.56 (m, 1H), 3.3–3.9 (m, 6H)

ESI-MS: *m/z* 588.7 $[M+H]^+$, ESI-MS/MS (–45 eV): *m/z* 334.4 $[M+H-4H_2O\text{-Acyl}]^+$

C_{16}-HFB_1: yield: 3.30 mg (70%)

^1H NMR (400 MHz, $CDCl_3$, ppm, *J* in Hz):

δ = 0.83 (d, *J* = 7.0, 3H), 0.87 (t, *J* = 6.6, 3H), 0.89 (t, *J* = 7.4, 3H), 0.99 (d, *J* = 7.0, 3H), 1.19 (d, *J* = 7.0, 3H), 1.24 (brs, 38H), 1.42 (brs, 6H), 1.8–2.0 (m, 3H), 2.18 (m, 2H),

2.34 (t, *J* = 7.4, 1H), 3.4 (dd, *J* = 5.16, 1H), 3.6–4.1 (m, 5H), 5.75 (d, *J* = 8.44, 1H)
ESI-MS: *m/z* 644.7 $[M+H]^+$, ESI-MS/MS (-47.5 eV): *m/z* 334.4 $[M+H\text{-}4H_2O\text{-Acyl}]^+$
$C_{24:1}$-HFB_1: yield: 3.76 mg (67%)
^1H NMR (400 MHz, $CDCl_3$, ppm, *J* in Hz):
δ = 0.82 (d, *J* = 6.5, 3H), 0.87 (t, *J* = 7.0, 3H), 0.89 (t, *J* = 7.0, 3H), 0.99 (d, *J* = 6.6, 3H), 1.19 (d, *J* = 6.6, 3H), 1.25 (m, 47H), 1.4–2.4 (m, 10H), 3.39 (dd, *J* = 2.9, 8.1, 1H), 3.6–4.0 (m, 4H), 4.03 (dd, *J* = 3.3, 8.5, 1H), 5.34 (brt, *J* = 4.6, 2H)
ESI-MS: *m/z* 754.8 $[M+H]^+$, ESI-MS/MS (–55 eV): *m/z* 334.4 $[M+H\text{-}4H_2O\text{-Acyl}]^+$

2.4 Synthesis of *N*-lauroyl, *N*-palmitoyl- and *N*-nervonoyl-HFB_2

The reaction was carried out as described above with HFB_2 (2 mg, 5.1 μmol), 1-hydroxybenzotriazole (HOBT) (0.8 mg, 5.7 μmol), EDC (1.1 mg, 5.8 μmol), 5.7 μmol fatty acid (1.1 mg lauric acid, 1.5 mg palmitic acid or 2.1 mg nervonic acid), and 2.0 mg *N,N*-diisopropylethylamine (15.4 μmol) in 0.3 mL of DMF. To eliminate any ester formation that occurred, the reaction mixture was stirred for 1 h with aqueous 1N NaOH at room temperature after extraction. For SiO_2 SPE cleanup the column was washed with 6 mL of $CHCl_3$, 2 mL of $CHCl_3$:MeOH (95:5, v/v) and eluted with 3 mL of $CHCl_3$:MeOH (95:5) and 3 mL of $CHCl_3$:MeOH (90:10) in 0.5-mL steps. The product-containing fractions were combined after identification by TLC and the solvent was removed.
C_{12}-HFB_2: yield 1.59 mg (54%)
^1H NMR (400 MHz, $CDCl_3$, ppm, *J* in Hz):
δ = 0.84 (d, *J* = 7.0, 3H), 0.87 (t, *J* = 7.0, 3H), 0.90 (t, *J* = 7.4, 3H), 0.94 (d, *J* = 6.6, 3H), 1.20 (d, *J* = 7.0, 3H), 1.0–1.8 (m, 43H), 1.25 (brs), 2.20 (t, *J* = 7.7, 1H), 3.37 (brd, J = 5.2, 1H), 3.45–4.05 (m, 4H)
ESI-MS: *m/z* 572.7 $[M+H]^+$, ESI-MS/MS (–35 eV): *m/z* 336.3 $[M+H\text{-}3H_2O\text{-Acyl}]^+$
C_{16}-HFB_2: yield 1.52 mg (47%)
^1H NMR (400 MHz, $CDCl_3$, ppm, *J* in Hz):
δ = 0.84 (d, *J* = 7.0, 3H), 0.87 (t, *J* = 7.0, 3H), 0.90 (t, *J* = 7.4, 3H), 0.94 (d, *J* = 6.6, 3H), 1.20 (d, *J* = 7.0, 3H), 1.0–1.4 (m, 50H), 2.21 (m, 2H), 3.36 (dd, *J* = 3.5, 8.5, 1H), 3.7–4.0 (m, 4H)
ESI-MS: *m/z* 628.7 $[M+H]^+$, ESI-MS/MS (–40 eV): *m/z* 336.3 $[M+H\text{-}3H_2O\text{-Acyl}]^+$
$C_{24:1}$-HFB_2 : yield 1.73 mg (46%)
^1H NMR (400 MHz, $CDCl_3$, ppm, *J* in Hz):
δ = 0.84 (d, *J* = 6.6, 3), 0.88 (t, *J* = 7.0, 3H), 0.90 (t, *J* = 7.0, 3H), 0.95 (d, *J* = 6.3, 3H), 1.27 (brs, 55H), 1.4-2.4 (m, 12H), 3.38 (dd, *J* = 3.6, 8.4, 1H), 3.5–3.8 (m, 3H), 4.05 (dd, *J* = 3.3, 8.4, 1H), 5.36 (brt, *J* = 4.6, 2H)
ESI-MS: *m/z* 738.8 $[M+H]^+$, ESI-MS/MS (–50 eV): *m/z* 336.3 $[M+H\text{-}3H_2O\text{-Acyl}]^+$

2.5 *In vitro* metabolism of $HFB_{1/2}$ by ceramide synthase

Ceramide synthase activity was assayed following acylation of either HFB_1 or HFB_2 with palmitoyl-CoA and nervonoyl-CoA. Typically, the reaction mixture contained 25 mM potassium phosphate buffer, pH 7.4, 0.5 mM DTT, 50 μM fatty acyl-CoA and varying concentrations of HFB_1 and HFB_2 (1 to10 μM, using aqueous stock solutions) in a total assay volume of 100 μL. The enzymatic reaction was started by adding 80 μg of microsomal protein (obtained from rat livers according to [34]) and placing the test tubes in a 37°C shaking water bath. After 15-min incubation, the reaction was stopped with 1 mL of MeOH:$CHCl_3$ (2:1) and 40 pomol of C_{12}-HFB_1 or C_{12}-HFB_2 was added as an internal standard for quantification. For product extraction, 1.25 mL of $CHCl_3$ was added, the mixture was vortexed and approximately 2 mL of slightly basic water was added. The layers were separated by centrifugation, the aqueous layer removed and the mixture washed two more times with basic water. The $CHCl_3$ layer was dried over Na_2SO_4 and evaporated to dryness using a Speed Vac. The residue was redissolved in 200 μL of $CHCl_3$:MeOH (90:10, v/v) and 10 or 20 μL thereof were subjected to HPLC-MS/MS analysis. For quantitative evaluation, the peak areas of the acylation products were compared with the areas of internal standards C_{12}-HFB_1/C_{12}-HFB_2. Due to the limited amount of HFB_x, only a limited number of data points could be measured to calculate the kinetic parameters.

2.6 Inhibition of ceramide synthase

The assay was conducted as described by Merrill and Wang [35], monitoring acylation of $[^3H]$sphingosine with palmitoyl- and nervonoyl-CoA in the presence of the *N*-acylated fumonisins. The total assay volume was 100 μL, containing 25 mM potassium phosphate buffer, pH 7.4, 0.5 mM DTT, 50 μM fatty acyl-CoA and either C_{16}-HFB_1, C_{16}-HFB_2, $C_{24:1}$-HFB_1 or $C_{24:1}$-HFB_2 (1 and 10 μM). In a control experiment ceramide formation was assayed in the absence of *N*-acyl compounds. Background radioactivity was determined in additional controls without fatty acyl-CoA. To eliminate the risk of adhesion of sphingosine to the walls of glass reaction vessels, the assay was performed in plastic tubes. The samples were incubated for 10 min at 37°C, the enzymatic reaction stopped by adding 1 mL of MeOH:$CHCl_3$ (2:1) and the mixture base-treated with 0.1N KOH for 1 h. After addition of ceramide as carrier, lipid extraction was done as described above. After solvent evaporation, the samples were redissolved in 20 μL of $CHCl_3$ and analyzed

by TLC. The activity of control cells was the same as has been reported earlier [18].

2.7 TLC analysis of ceramide synthase assay products

The samples were spotted onto silica plates that were developed with diethylether:MeOH (99.5:0.5, v/v). Radioactivity was detected by exposing the plates to a photographic film for 24 to 48 h at −80°C. The radioactive ceramide regions were collected and counted using a detergent-containing scintillation mixture for 10 min in a Beckman LS 7000 scintillation counter.

2.8 *In vitro* cytotoxicity

HT29 cells (ATCC, Rockville, MD) were seeded at 1×10^5 cells per 100-mm dish (Corning, Cambridge, MA), and grown in 8 mL of DMEM (Sigma) supplemented with 10% fetal calf serum (HyClone, Logan, UT), 3.5 g/L glucose, and 10 mL/L of a solution of 6.1 mg/mL penicillin G and 10 mg/mL streptomycin (Sigma) in an incubator at 37°C and a humidified atmosphere of 5% CO_2. The medium was changed 24 h after seeding, then the cells were incubated with DMEM containing *N*-acylated aminopolyols (solubilized in 10 mL of ethanol:dodecane, 98:2; addition of this volume of ethanol:dodecane had negligible effects on cell viability). After 24 h, the cells were harvested with 0.25% trypsin in 0.05% EDTA. The number of viable cells remaining was assessed using the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)] assay.

2.9 *In vivo* metabolism of HFB_1

The experimental protocol for the *in vivo* study was approved by the Animal Care and Use Committee, Richard B. Russell Agricultural Research Center, Agricultural Research Service-USDA, Athens, GA. Male Sprague-Dawley rats (Harlan, Indianapolis) were acclimated (10 days) and then randomly assigned to groups of two (controls) to three (all other groups) animals each. Rats were individually housed in stainless steel, wire-mesh cages in an environment-controlled facility (12/12 light/dark cycle) at the Richard B. Russell Agricultural Research Center, USDA. A standard rodent diet (LM-485, Teklad, Madison, WI) and tap water were provided *ad libitum*. The animals were 6 weeks of age and group mean body weights ranged from 196 (±4.6 SD) to 198 (4.2) g at the start of the study (day 1).

Animals were given intraperitoneal injections of sterile, physiological saline (controls) or HFB_1 dissolved in sterile saline on 5 consecutive days. HFB_1 concentrations of the dosing solutions were determined by routine HPLC using OPA derivatization and fluorescence detection as described in [24]. Groups and treatments are given in the following table (Table 1).

Table 1. Experimental design of the animal study

Group	Daily dose μg HFB_1	μg HFB_1/kg BW[e)]	Total dose[f)] μg HFB_1
Control	0[a)]	0	0
Low dose	52[b)]	253 ± 5	260
Mid dose	115[c)]	558 ± 9	575
High dose	230[d)]	1120 ± 11	1150

a) Sterile saline, injection volume = 5 mL.
b) HFB_1 concentration in dosing solution = 10.4 μg/mL, injection volume = 5 mL.
c) HFB_1 concentration in dosing solution = 115 μg/mL, injection volume = 1 mL.
d) HFB_1 concentration in dosing solution = 115 μg/mL, injection volume = 2 mL.
e) BW, body weight; value indicates mean ± SD; amount injected per day/average BW of the animals during the 4-day dosing period.
f) Total amount of HFB_1 administered over 4 days.

Injection sites were rotated daily to minimize irritation. The animals were observed daily and weighed at study initiation and on day 4 (before fasting). Food consumption from study initiation through day 4 was monitored. The animals were fasted overnight prior to the last injection (day 5). Approximately 2 h after the last dose was given, both controls and two animals each from the three dosed groups were anesthetized and blood samples (approximately 2 mL, taken from the periorbital sinus) collected and stored at –80°C. The rats were then euthanized (CO_2 inhalation followed by exsanguination via the portal vein) and necropsied. The brain, gastrointestinal tract and representative samples of heart, kidney, liver and fat were collected and stored (–80°C) for analyses of *N*-acylated hydrolyzed fumonisins or quantification of sphingoid bases (liver and kidney). Additional liver and kidney samples were fixed in 10% neutral buffered formalin. After fixation, they were embedded in paraffin, sectioned (4 microns), mounted on glass slides and stained with hematoxylin and eosin for microscopic examination. The remaining low-dose, mid-dose and high-dose rats were observed and weighed weekly for an additional 3 weeks. At the end of this"wash out" period, they were killed and tissue samples collected as described above.

2.10 Extraction of tissues

Samples (100 mg of tissue) were homogenized in 0.9 mL of PBS and 0.2-mL aliquots were transferred to four glass test tubes (16 6 100 mm). To extract the lipids 2.4 mL of $CHCl_3$:MeOH (1:2) and standards (0.5 nmol of each of C_{20}-sphingosine, C_{12}-ceramide and C_{12}-HFB_1) was added. The mixture was vortexed, 2.4 mL of $CHCl_3$ and 5 mL of H_2O were added to split into two phases, the mixture was vortexed again and centrifuged. The $CHCl_3$ layer was removed and the aqueous phase was re-extracted three times, each with 1 mL of $CHCl_3$. The combined $CHCl_3$ extracts were

dried, evaporated to dryness using a Speed Vac, dissolved in 0.1 mL of 0.1 M KOH in methanol, and incubated for 2 h at 37°C. One microliter of $CHCl_3$ was added, the mixture was centrifuged to remove insoluble material, then transferred to a new glass test tube, dried using a Speed Vac and dissolved in 200 µL of solvent A for HPLC-MS/MS analysis.

2.11 Statistics

All analyses were conducted at least in triplicate and the results are given as mean values ± SE. The statistical significance of differences was determined by the unpaired Student's *t*-test using SigmaPlot. The kinetic parameters were derived from linear regression analyses of the data.

3 Results

As HFB_1 is known to be acylated by ceramide synthase using palmitoyl-CoA, the initial experiments investigated the acylation of HFB_1 and HFB_2 to determine if both types of fumonisins were handled this way as well as compared palmitoyl (C_{16})-CoA and nervonoyl ($C_{24:1}$)-CoA as the fatty acyl-CoA cosubstrate. Nervonoyl-CoA was chosen due to its relative abundance within the very-long-chain ceramide fraction of sphingolipids [35, 36] as well as because its utilization would indicate that more than one CerS isoform is able to acylate these compounds [29].

3.1 Analysis of acylation products by HPLC-MS/MS

A new method based on HPLC-ESI-MS/MS was developed for the detection and qualitative and quantitative analysis of the $HFB_{1/2}$ acylation products. The possibly formed products *N*-palmitoyl- and *N*-nervonoyl-HFB_1 (C_{16}/$C_{24:1}$-HFB_1) and -HFB_2 (C_{16}/$C_{24:1}$-HFB_2) as well as *N*-lauroyl-HFB_1 and -HFB_2 (C_{12}-$HFB_{1/2}$), for use as internal standards, were synthesized (structures see Fig. 1), their fragmentation in the MS/MS spectrometer were studied and the characteristic product ions were used for the setup of suitable MRM experiments. The electrospray mass spectra of all compounds showed the protonated molecular ions $[M+H]^+$ at *m/z* 588.7, 644.7 and 754.8 for C_{12}-HFB_1, C_{16}-HFB_1 and $C_{24:1}$-HFB_1 and at *m/z* 572.7, 628.7 and 738.8 for C_{12}-HFB_2, C_{16}-HFB_2 and $C_{24:1}$-HFB_2. Fragmentation of the protonated molecular ions resulted in the cleavage of the fatty acyl chain and successive loss of several water molecules, thus yielding an $[M+H-4H_2O\text{-acyl}]^+$ ion at *m/z* 334.4 as the predominant product ion for the HFB_1 series. Figure 2 shows a product ion spectrum of $C_{24:1}$-HFB_1 with the typical fragmentation yielding the characteristic ion at *m/z* 334.4. The HFB_2 compounds showed an $[M+H-3H_2O\text{-acyl}]^+$ at *m/z* 336.3 as the major fragmentation product. These characteristic product ions were employed for MRM experiments.

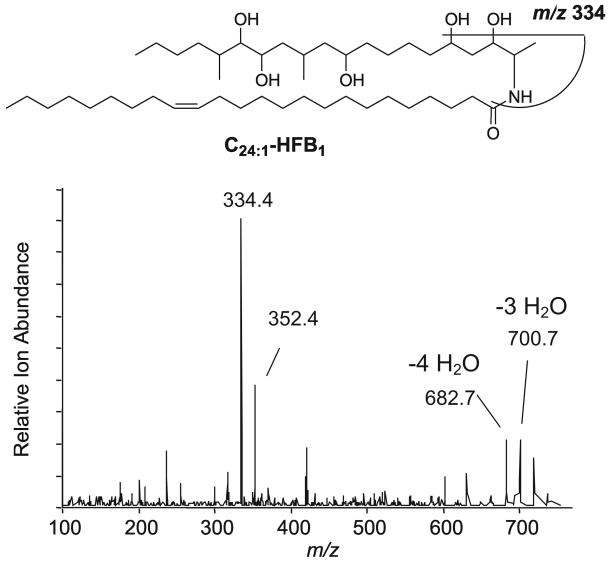

Figure 2. Product ion spectrum and fragmentation of *N*-nervonoyl-HFB_1 ($C_{24:1}$-HFB_1) (collision gas N_2, offset voltage 35 eV).

The analysis of sample material required the separation of the acylation products from interfering matrix components. For this purpose, chromatographic methods had to be developed for both the HFB_1 and HFB_2 series. Quantitative evaluation was carried out by comparison of the peak areas with those of the internal standards C_{12}-HFB_1 and C_{12}-HFB_2 after validation that under the optimized MRM conditions each gave similar ion yields. With an LOD of about 10 fmol/µL the sensitivity of the developed method was well sufficient for a reliable analysis.

3.2 Acylation of HFB_1 and HFB_2 by ceramide synthase(s)

Incubation of rat liver microsomes with HFB_1 or HFB_2 and either nervonoyl- or palmitoyl-CoA resulted in the formation of the respective acylation product, namely *N*-nervonoyl-HFB_1 ($C_{24:1}$-HFB_1) and -HFB_2 ($C_{24:1}$-HFB_2) and *N*-palmitoyl-HFB_1 (C_{16}-HFB_1) and -HFB_2 (C_{16}-HFB_2), all of which were unambiguously identified in the MRM experiments via their protonated molecular ions and characteristic product ions. Most remarkably, a comparison of the occurring product amounts showed that acylation of HFB_1 as well as HFB_2 is faster with nervonoyl-CoA than with the palmitoyl coenzyme. Figure 3 illustrates the amounts of acylation products obtained at two different substrate concentrations. In the case of HFB_1, five- and tenfold more $C_{24:1}$-HFB_1 than C_{16}-HFB_1 was formed at 2 and 10 µM HFB_1, respectively. Similarly, the formation of $C_{24:1}$-HFB_2 exceeded that of C_{16}-HFB_2 by factors of 3 (2 µM) and 7 (10 µM). HFB_1 and HFB_2 were equally well accepted as substrates by ceramide synthase, hence, the results are expressed for "$HFB_{1/2}$" where the findings apply to both.

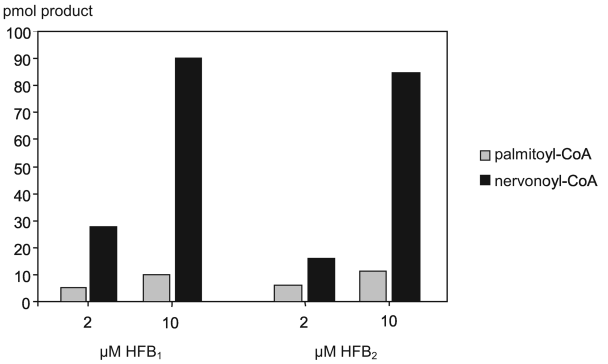

Figure 3. Amounts of acylation products obtained by incubation of rat liver microsomes with nervonoyl- and palmitoyl-CoA and HFB_1 or HFB_2 at two different concentrations.

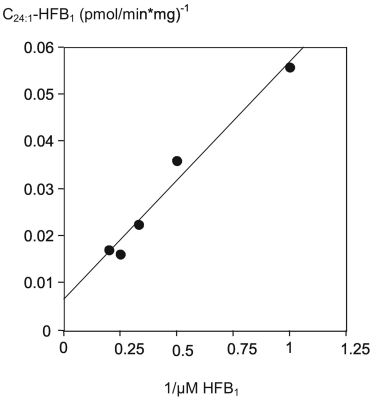

Figure 4. Double reciprocal plot of acylation of HFB_1 by rat liver microsomes using varying concentrations of HFB_1 and 50 μM nervonoyl-CoA.

The formation of $C_{24:1}$-HFB_1 and C_{16}-HFB_1 followed a simple Michaelis-Menten relationship. A double reciprocal (Lineweaver-Burk) plot was chosen for linearization in order to determine the characteristic kinetic parameters. For $C_{24:1}$-HFB_1 formation, the apparent K_m was 7.9 μM and v_{max} was 157 pmol/min/mg microsomal protein (Fig. 4), whereas the acylation of HFB_1 with palmitoyl-CoA occurred with a K_m of 1.2 μM and at a v_{max} of 8.7 pmol/min/mg protein (Fig. 5). Expressed as v_{max}/K_m, the formation of $C_{24:1}$-HFB_1 (19.9) was approximately twofold higher than formation of $C_{16}HFB_1$ (7.3). Unfortunately, it was not possible to determine K_m and v_{max} for the acylation of HFB_2 due to the limitated availability of substrate.

3.3 Cytotoxicity of *N*-acylated hydrolyzed fumonisins to HT29 cells

The cytotoxicity of the ceramide synthase acylation products C_{16}-HFB_1, $C_{24:1}$-HFB_1, C_{16}-HFB_2 and $C_{24:1}$-HFB_2 was determined by incubation of each compound with HT29 cells, a human colonic cell line sensitive to FB_1 and HFB_1 [23]. Figure 6 summarizes the results. All substances sig-

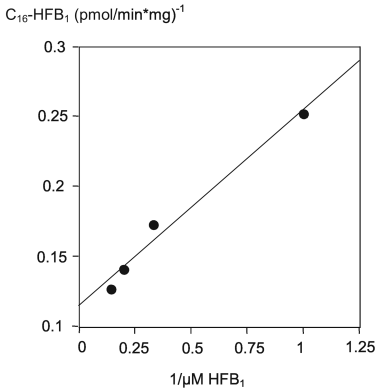

Figure 5. Double reciprocal plot of acylation of HFB_1 by rat liver microsomes using varying concentrations of HFB_1 and 50 μM palmitoyl-CoA.

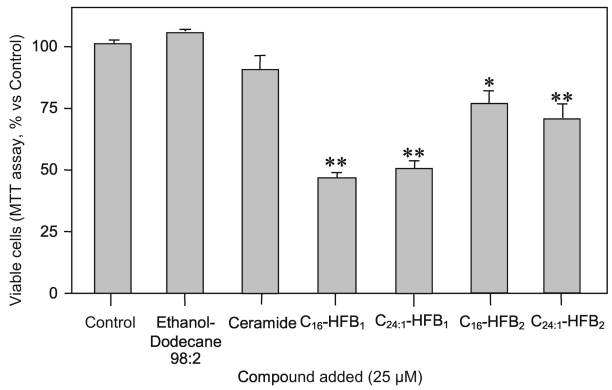

Figure 6. Toxicity of *N*-acylated aminopolyols on HT29 cells at a concentration of 25 μM (*$p < 0.05$; **$P < 0.01$; *vs.* ethanol:-dodecane 98:2). The results are shown as mean ± SE ($n = 3$) (Control: cell medium, ethanol-dodecane: control containing the solvent carrier, ceramide: control with 25 μM C2-ceramide).

nificantly reduced the number of viable cells at a concentration of 25 μM. After 24-h incubation, C_{16}-HFB_1 and $C_{24:1}$-HFB_1 caused a 50% reduction in the number of viable cells and were therefore more potent than C_{16}-HFB_2 and $C_{24:1}$-HFB_2, which caused a 30% reduction. Although the MTT assay cannot distinguish between reduced cell viability due to cytotoxicity and lower cell numbers due to growth inhibition, the results clearly demonstrate that the *N*-acylated metabolites are more potent compared to FB_1 and HFB_1 [23, 28].

3.4 Inhibition of ceramide synthase by *N*-acylated hydrolyzed fumonisins

Because inhibition of ceramide synthase(s) appears to be a key step in the toxicity of fumonisins, we also evaluated the acylation products' ability to inhibit ceramide formation. Thus, the ceramide synthase-mediated acylation of [^{3}H]sphingosine with palmitoyl- or nervonoyl-CoA was

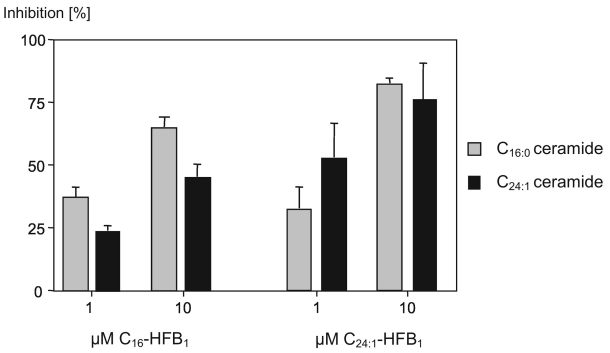

Figure 7. Inhibition of ceramide synthase activity, measured using the acylation of [^{3}H]sphingosine, by C_{16}-HFB_1 and $C_{24:1}$-HFB_1 at 1 and 10 µM. The data are shown as the mean ± SE (n = 3–5).

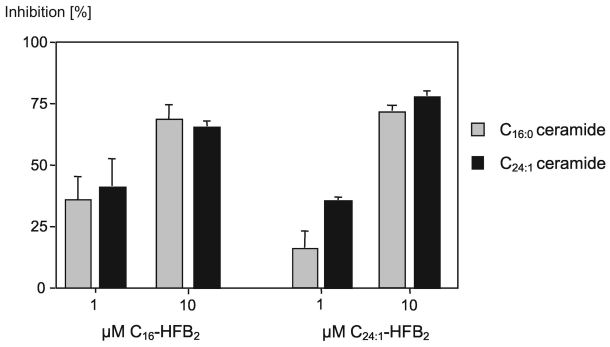

Figure 8. Inhibition of ceramide synthase activity, measured using the acylation of [^{3}H]sphingosine, by C_{16}-HFB_2 and $C_{24:1}$-HFB_2 at 1 and 10 µM. The data are shown as the mean ± SE (n = 3–5).

monitored in the presence of 1 and 10 µM $C_{24:1}$-HFB_1, $C_{24:1}$-HFB_2, C_{16}-HFB_1 or C_{16}-HFB_2. At concentrations of 1 µM, the HFB_1 compounds inhibited ceramide synthase activity by 30% (Fig. 7) while up to 80% inhibition was observed at 10 µM. C_{16}-HFB_2 and $C_{24:1}$-HFB_2 acted similarly, causing 30 and 70% inhibitions at 1 and 10 µM, respectively (Fig. 8). These findings are in agreement with the results of the cytotoxicity tests, suggesting that the acylated hydrolyzed fumonisins might exert toxic effects via a similar mode of action as FB_1.

3.5 *In vivo* acylation of HFB_1 and HFB_2

Because HFB_1 and HFB_2 are acylated *in vitro* by the ceramide synthase(s) to form *N*-acyl-metabolites with cytotoxic and ceramide synthase inhibitory potential, the formation of these metabolites was investigated *in vivo*. Blood and tissues from rats that had been administered HFB_1 (at 52, 115 and 230 µg/day, intraperitoneal) for 5 days were extracted as described in Section 2.10 and analyzed by HPLC-MS/MS. Interestingly, several *N*-acyl-metabolites were found. Figure 9 shows an HPLC-MRM-MS chromatogram of a rat

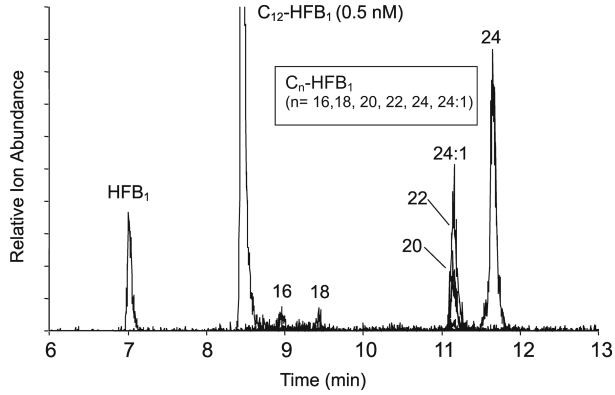

Figure 9. HPLC-MRM-MS chromatogram of a rat liver extract from an animal of the high-dose group (230 µg HFB_1/day). Several *N*-acyl-HFB_1-metabolites (C_n-HFB_1) with fatty acids of various chain length could be detected (C_{12}-HFB_1: internal standard) (structures see Fig. 1).

liver extract from an animal of the high dose group (230 µg HFB_1/day). Besides residual HFB_1 and the C_{12}-HFB_1 internal standard, HFB_1 acylation products with fatty acids of various chain lengths were unambiguously identified. The most prevalent species found were the HFB_1-acyl compounds containing long-chain fatty acids (C_{24}, $C_{24:1}$, C_{22} and C_{20}). Lesser amounts of the respective metabolites with C_{16} and C_{18} fatty acids were also detected (structures see Fig. 1).

Acylation products were not found in the control animals, indicating that their formation occurred as a result of the exposure to HFB_1. In addition, no *N*-acyl-metabolites were found in the animals analyzed three weeks after the last dose was given, indicating that the acylated-$HFB_{1/2}$ metabolites were further metabolized and/or excreted. The quantitative evaluation of the data from the individual rats of the three dose groups is summarized in Fig. 10. The liver and kidney (data not shown) of all animals had a similar distribution pattern for the fatty acids found in the metabolites, with the C_{24}-compound occurring in the highest amounts (up to 2.4 nmol/g), followed by $C_{24:1}$-HFB_1 (up to 0.8 nmol/g) and C_{22} (0.25 nmol/g). C_{16}-HFB_1 and C_{18}-HFB_1 were detected in only a few of the animals, and in those cases typically at the order of 0.04 to 0.13 nmol/g (with the highest amounts being 0.17 and 0.54 nmol/g, respectively). The low amounts of the latter metabolites were not correlated with the dose and therefore were probably due to variations in ceramide synthase subtypes among individual animals.

Gross and microscopic examinations of the liver and kidney, which are the major target organs of fumonisins in rats, from the animals killed on day 5 did not reveal any treatment-related alterations. Body weight, food consumption, and kidney and liver weights of all groups were likewise similar (data not shown). Thus, the intraperitoneal (ip) doses used in this short-term experiment, which ranged up to 1.12 mg/kg body weight, were insufficient to cause overt tissue damage. The bioavailability of HFB_1 is not known

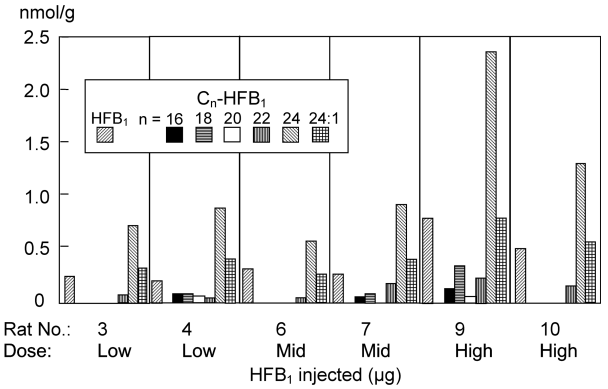

Figure 10. Distribution of *N*-acyl-HFB_1-metabolites (C_n-HFB_1) of various fatty acid chain lengths in the livers of six rats that had been given low [52 µg/kg body weight (BW)], mid (115 µg/kg BW) or high (230 µg/kg BW) intraperitoneal (ip) doses of HFB_1 for 4 consecutive days. No *N*-acyl-HFB_1-metabolites were found in the livers of control animals (rats No. 1 and 2) or in rats 5 (low dose), 8 (mid dose) and 11 (high dose), which were killed 3 weeks after the last day of dosing (structures see Fig. 1).

but is considerably higher than that of FB_1 [37]. Therefore, the dietary HFB_1 concentrations corresponding to our ip doses cannot be calculated but could have ranged from 10 ppm (if gastrointestinal absorption approaches 100%) to about 200 ppm (bioavailability of 5%) at the high-dose level (Group B2). An intermediate value is likely [37].

4 Discussion

Recently, we identified *N*-palmitoyl-HFB_1 (C_{16}-HFB_1) as the cytotoxic product from the acylation of HFB_1 with palmitoyl-CoA by ceramide synthase *in vitro* [28]. We have now identified *N*-nervonoyl-HFB_1 ($C_{24:1}$-HFB_1), *N*-palmitoyl- and *N*-nervonoyl-HFB_2 (C_{16}-HFB_2, $C_{24:1}$-HFB_2) as new metabolites formed by ceramide synthase with hydrolyzed fumonisins HFB_1 and HFB_2 *in vitro*. Acylation apparently does not detoxify hydrolyzed fumonisins because C_{16}-HFB_1, C_{16}-HFB_2, $C_{24:1}$-HFB_1 and $C_{24:1}$-HFB_2 metabolites were cytotoxic for HT29 cells as well as inhibited ceramide synthase *in vitro*.

These results demonstrate that HFB_1 and HFB_2 are acylated *in vitro* and *in vivo*. It is likely that the enzyme(s) that are responsible for acylation are ceramide synthases because $HFB_{1/2}$ are inhibitors of sphingosine acylation as well as themselves acylated, as expected for substrate competitive inhibitors. If so, it remains intriguing that even though these compounds differ from the natural substrates sphinganine and sphingosine in the stereochemistry at C2 and C3 (the carbon atoms in the vicinity of the group to be modified) and other structural elements (the absence of the hydroxyl group at C1 and the presence of an hydroxyl function at C5), the enzyme(s) are still able to use HFB_1 and HFB_2 as substrates. This agrees with results of studies using various stereoisomers of sphinganine and synthetic 1-deoxy-5-hydroxy-sphinganines as substrates for ceramide synthase that have concluded that both *erythro* and *threo* isomers are reasonably well accommodated by these enzymes [28, 38].

A comparison of the apparent v_{max} showed that acylation with nervonoyl-CoA is 18-fold faster than with palmitoyl-CoA, or >2-fold better utilized when v_{max}/K_m are compared. Importantly, HPLC-MS/MS analysis of the liver samples from rats dosed with HFB_1 has demonstrated for the first time that *N*-acyl-HFB_1-metabolites (C_n-HFB_1) with fatty acids of various chain lengths (C_{16}, C_{18}, C_{20}, C_{22}, C_{24} and $C_{24:1}$) are also formed *in vivo*. The preferred acylation by $C_{24:1}$-fatty acyl-CoA *in vitro* and the finding that mainly very-long-chain *N*-acyl-HFB_1-metabolites are formed *in vivo* (Figs. 9 and 10) may be interpreted in the context of recent findings that mammals have multiple ceramide synthase genes (*Lass* 1–6 recently named CerS1-6), showing a different specificity concerning the fatty acyl-CoA (for a review see [29]). It was first shown that overexpression of LASS1 (CerS1) resulted in a selective increase in C18-ceramide in mammalian cells [30]. Subsequently, LASS4 (CerS4) and LASS5 (CerS5) were shown to selectively utilize $C_{18/20}$ and C_{16} acyl-CoAs, respectively [31, 32], LASS6 to produce shorter chain ceramides (C_{14} and C_{16}) [39] and LASS3 to produce very-long-chain ceramides (C_{18}, C_{24}) (and LASS2, CerS2, appears to be relatively nonspecific) [40]. It is not known why mammals have multiple CerS genes, but it implies an important role for ceramides containing specific fatty acids. The preferred formation of very-long-chain *N*-acyl-HFB_1-metabolites – as shown in the liver (Figs. 9 and 10) – implies that CerS2 and CerS3, which appear to be most active with the corresponding fatty acyl-CoA, may be the enzyme(s) that are most involved in acylation of the hydrolyzed fumonisins, either because these can best accommodate the $HFB_{1/2}$ molecules or perhaps due to these being the major LASS (CerS) genes that are expressed in the tissues. Future studies could use genetic approaches, such as suppression of individual LASS (CerS) proteins using siRNA, to test this hypothesis. Studies on the toxic effects of hydrolyzed fumonisins have yielded mixed results. Liver and kidney lesions and liver tumor promoting activity have been demonstrated in rats fed hydrolyzed *Fusarium* culture materials containing hydrolyzed fumonisins, but no detectable (HPLC) FB_1 or FB_2 [24, 25]. In these studies, the potency of HFB_1 was about equal to or less than that of FB_1. In contrast, Collins *et al*. [27] reported that HFB_1 did not induce tissue lesions or affect sphingoid bases when given orally to pregnant rats. Howard *et al*. [26] also found no evidence that HFB_1 was toxic when fed at concentrations of ca. 29 and 58 ppm (corresponding to 70 and 140 µmol/kg diet, respectively) to female mice for 28 days. In contrast, FB_1 caused hepatic lesions and affected sphingolipid metabolism at these (µmol/kg diet) concentrations. The reason that the findings of Hendrich *et al.* [25] and Voss *et al.* [24] do not

agree with those of Collins *et al.* [27] and Howard *et al.* [26] is not known. One possibility is that matrix-bound or "hidden" FB_1, which could become bioavailable during digestion, may have been present in the culture materials (reviewed in [41]). Differences in dosing protocols and or physiological differences among the species and, within species, the strains of animals used in the experiments are also possible. The latter could include differences in ability of the animals to acylate the hydrolyzed fumonisins. Therefore, studies comparing the metabolism of HFB_1 in rats and mice would be of interest because they might reveal that the reported differences in sensitivity of mice and rats to HFB_1 might depend on the extent and type of metabolism.

There is evidence indicating that fumonisins are potential risk factors for cancer or neural tube (birth) defects in humans but an unequivocal relationship between human disease and exposure to fumonisins or hydrolyzed fumonisins has not been established. However, that HFB_1 and the acylation products are reducing the number of viable HT29 cells, suggests that consumption of fumonisins or hydrolyzed fumonisins might adversely affect the human gastrointestinal tract or other tissues. Following oral administration (gavage) of FB_1 to non-human primates, a portion of the dose recovered in the intestinal epithelial cells, excreta, bile and gut contents consisted of HFB_1 and partially hydrolyzed FB_1 [42] and, based on our cytotoxicity results, hydrolyzed fumonisins or their metabolites might damage the gut epithelium. Furthermore, according to one report [37], the bioavailability of HFB_1 in rats is greater than that of FB_1, increasing the likelihood that HFB_1, either injested via contaminated alkali-processed corn products [41] or formed by hydrolysis of FB_1 in the gastrointestinal tract can be absorbed by the gut and thereby allow subsequent metabolism to $C_{24:1}$-HFB_1 and C_{16}-HFB_1 in the liver or other tissues to enhance its biological effects.

In summary, we have demonstrated that HFB_1 and HFB_2 are metabolized by microsomes *in vitro* and (at least) HFB_1 by rats *in vivo* to acylated derivatives that resemble ceramide in structure. These compounds reduce the number of viable HT29 cells and inhibit ceramide synthase and, as a result, warrant further investigations on the role they might play in the toxicity of hydrolyzed fumonisins.

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