Instability of N-acetylated fumonisin B1 (FAI) and the impact on inhibition of ceramide synthase in rat liver slices

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Abstract

Fumonisin B1 (FBI) is a mycotoxin produced by Fusarium verticillioides. It inhibits ceramide synthase, which is a proposed underlying mechanism responsible for the myriad of toxic endpoints observed. We previously reported that N-acetylation of FBI prevents ceramide synthase inhibition, but cautioned that impure preparations of FAI can contain a contaminant with the ability to inhibit ceramide synthase. We now report that FAI spontaneously rearranges to O-acetylated analogs. These rearrangement products are putative inhibitors of ceramide synthase. Rat liver slices exposed to impure FAI containing O-acetylated FBI had sphinganine/sphingosine (Sa:So) ratios of 1.15-1.64. Control slices had Sa:So ratios of 0.07-0.24. Clean-up to remove the O-acetylated FBI yielded purified FAI, which produced Sa:So ratios in liver slices of 0.08-0.18. After storage for approximately 1 year as either a dry powder in a desiccator, or as a dried film at 4°C, the purified FAI again contained O-acetylated FBI, and was capable of ceramide synthase inhibition. FAI was most stable in neutral solution, but in acidic solution the equilibrium shifted towards the O-acetylated forms. FAI in solid form also rearranged, but more slowly than in acid solution. As FAI is considerably less cytotoxic than FBI, these results provide additional support for the conclusion that a primary amino group is necessary for both ceramide synthase inhibition and toxicity. Published by Elsevier Science Ltd.

Keywords: Fumonisin; Acetylated fumonisin; Mycotoxin; Ceramide synthase; Sphingolipid; Rat liver slices

1. Introduction

Fumonisins are a family of structurally related toxic fungal metabolites produced by several Fusarium species (Fig. 1). They are most commonly found as contaminants of corn, where they are produced by the corn endophyte, F. verticillioides (syn. F. moniliforme) (Bacon and Hinton, 1996). Consumption of fumonisin-contaminated corn can cause fatal diseases in horses and pigs, is suspected of causing health and performance problems in poultry and other domesticated animals, and has been linked to esophageal cancer in some parts of the world (EHC, 2000). Recently completed studies by the US National Toxicology Program confirmed that fumonisin B1, the predominant naturally occurring analog, is carcinogenic to rats and mice (Howard et al., 2001). Fumonisins are structurally similar to sphingoid bases, and were determined to be potent inhibitors of sphinganine N-acyl transferase (ceramide synthase) (Wang et al., 1991). Research since this discovery has provided substantial evidence that the toxicity and carcinogenicity of fumonisins is related to the disruption of sphingolipid metabolism that occurs as a result of inhibition of ceramide synthase (Riley et al., 1993, 1994, 1998; Merrill, et al., 1995).

Four analogs of fumonisin were originally isolated from F. verticillioides-inoculated corn—A1, A2, B1 and B2 (Bezuidenhout et al., 1988). Several reports have demonstrated the reduced toxicity of N-acetylated fumonisins relative to FBI or other fumonisins with primary amino groups ( Abbas et al., 1993; Gelderblom et al., 1993). Merrill et al. (1996) reported that FAI had less than 2% of the ceramide synthase inhibiting ability of FBI in in vitro experiments, even at a dose of 10 µM. Later, we studied the ability of a number of fumonisin analogs, including FAI, to inhibit ceramide synthase in rat liver slices (Norred et al., 1996). Our findings
confirmed that the primary amino group is necessary for inhibition of the enzyme, but also revealed that preparations of FA1 could contain an unknown contaminant that had ceramide synthase inhibitory activity, which must be removed to prevent a false positive response. All of these findings are consistent with the hypothesis (Riley et al., 1998) that toxicity and disruption of sphingolipid metabolism by fumonisins are linked.

A recent study by Van der Westhuizen et al. (1998) investigated the ability of FB1 and several structurally related compounds to inhibit sphingolipid biosynthesis, and concluded that FA1 was equipotent to FB1 as a ceramide synthase inhibitor, contradicting the above reports. As they had earlier shown that FA1 was less cytotoxic than FB1 (Gelderblom et al., 1993), it was concluded that cytotoxicity of fumonisins is not solely due to ceramide synthase inhibition and disruption of sphingolipid metabolism. In the present study, we report that the N-acetylated analog of fumonisin B1 can spontaneously rearrange to O-acetylated derivatives. When these contaminants are present, ceramide synthase activity in rat liver slices is inhibited. Clean-up of the preparation to remove the O-acetylated compounds yields highly purified FA1, which no longer inhibits sphingolipid biosynthesis. These results support the necessity of a primary amino group for both toxicity and ceramide synthase inhibition by fumonisins.

2. Materials and methods

2.1. Mycotoxins

FB1 and FA1 were isolated from corn cultured with F. verticillioides MRC 826 and purified by previously described methods (Poling and Plattner, 1999; Meredith, 2000). FA1 was subjected to additional clean-up procedures as follows, and as illustrated in Fig. 2. A crude preparation (140 mg) from an extract of F. verticillioides MRC 826 that contained mostly FA1, but also FB1 and other contaminants, was dissolved in 200 ml of 50:50 CH3CN/H2O. The solution was passed through a 10-g strong cation exchange (SCX) Mega Bond Elut cartridge [40 µm, 1225-6035, Varian Sample Preparation Products, Harbor City, CA, USA] that was preconditioned with 100 ml methanol followed by 100 ml 50:50 CH3CN/H2O. An additional 50 ml of 50:50 CH3CN/H2O was passed through the cartridge and combined with the initial eluate. FB1 was retained on the cartridge, whereas FA1 and other contaminants were eluted. A 10-g NH2 cartridge (Sep-Pak Vac 35 cc (10 g) NH2 cartridge, WAT043350) was conditioned with 100-ml aliquots of methanol and then 50:50 CH3CN/H2O. The eluate from the SCX cartridge was applied to the NH2 cartridge and the cartridge washed with 100-ml aliquots of 50/50 and 10/90 CH3CN/H2O. The cartridge was washed successively with 100-ml aliquots of 5% acetic acid in (1) 10/90, (2) 20/80, (3) 30/70, and (4) 40/60 CH3CN/H2O. To remove acetic acid, eluates that contained FA1 (2, 3 and 4) were diluted with H2O to a final solvent composition of 10/90 acetonitrile/water. Then the fractions were applied to 10-g tC18 cartridges [Sep-Pak Vac 35 cc (10 g) tC18 Cartridge, WAT043350, Waters Corp., Milford, MA, USA] preconditioned with 100 ml methanol and then 100 ml 10/90 CH3CN/H2O. The cartridge was washed (the void volume was pulled through each time) with 100 ml 10/90. FA1 was eluted in two 50-ml fractions with 50/50 acetonitrile/water. Only highly purified FA1 (80 mg) from eluate 3 was used for biological testing. The above clean-up procedure was also applied to re-purify FA1 preparations that had rearranged to O-acetyl derivatives.

2.2. Ceramide synthase assay

Precision-cut rat liver slices, obtained from male Sprague–Dawley rats (200–300 g, Harlan Industries, Indianapolis, IN, USA), were used to assay solutions of FB1 and FA1 for their ability to inhibit ceramide synthase as previously described (Norred et al., 1996). The mycotoxins were dissolved in water, and liver slices in 1.0-ml Weymouth's medium (Gibco, Grand Island, NY, USA) were dosed with 10 µl of solution to yield final concentrations of toxin of 0.5 or 5.0 µM. After incubation at 37°C for 24 h, the slices were washed with Hanks' buffered salt solution (Gibeo, Grand Island, NY, USA) and processed for measurement of content of free sphingosine and free sphinganine as indicators of

Fig. 1. Structures of fumonisins and sphinganine. TCA, tricarballylic acid; 3-OAF, 3-O-acetyl fumonisin B1; 5-OAF, 5-O-acetyl fumonisin B1.
A SpectraSYSTEM P4000 pump was coupled to a LCQ mass spectrometer via an electrospray interface (ESI) (Thermoquest-Finnigan, San Jose, CA, USA). An Intersil ODS-3 15 cm x 3.0 mm ID column (0395-150×030, MetaChem Technologies, Torrance, CA, USA) was used and the entire HPLC eluent was introduced into the MS-detector. A 10-min linear gradient from 65/35/00 to 5/35/60 water/1% acetic acid in methanol/methanol was used and the final composition was held for 20 min. The flow rate was 0.3 ml/min. Mass spectra were obtained by scanning from m/z 300 to 950. The ESI spray voltage was 4.5 kV and the capillary temperature was 220°C. The sheath gas was nitrogen with the flow rate set to 65 arb (Thermoquest-Finnigan term for “arbitrary ml/min”, since the flow cannot be precisely calibrated in this region of the HPLC–MS interface).

2.3. Statistical analysis

Three replicate experiments were conducted for each toxin and dose tested, and the results were analyzed by analysis of variance procedures using a commercially available computer program (SigmaStat, Jandel.

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Fig. 2. Scheme for purification of N-acetylated fumonisin B1 (FA1) using strong cation exchange (SCX), NH2, and C18 cartridges.
Scientific, San Rafael, CA, USA). When significant differences were detected, Dunnett's method was used to identify values that were significantly different from control values, with a significance level of $P < 0.05$.

3. Results

Significant elevations occurred in sphinganine content and the ratio of sphinganine to sphingosine (Sa/So) in rat liver slices exposed to fumonisins B1 or impure FA1 preparations period (Table 1). No differences were found in the quantity of sphingosine in slices in any of the treatment groups compared to control slices. Subsequent analysis of the impure FA1 preparation revealed the presence of contaminants with retention times of 12.7 and 13.2 min, compared to a retention time of 16.2 min for N-acetylated FB1 (FA1) (Fig. 3). The contaminating peaks were identified as 3-O-acetyl FB1 and 5-O-acetyl FB1 based on their chromatographic retention times, their ESI-MS spectra, the kinetic order of rearrangement observed on heating in aqueous acetic acid and their chromatographic properties on SCX and weak anion exchange columns. Both FA1 and the contaminants had an apparent molecular weight of 763 but had markedly different ESI-MS spectra. The spectrum of FA1 showed a protonated molecule at m/z 764 along with adduct ions with one

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sphingosine (So) (pmol/slice)</th>
<th>Sphinganine (Sa) (pmol/slice)</th>
<th>Ratio (Sa/So)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.01±0.87</td>
<td>0.75±0.43</td>
<td>0.15±0.08</td>
</tr>
<tr>
<td>FB1 (0.5 μM)</td>
<td>4.98±2.13</td>
<td>6.36±3.47</td>
<td>1.25±0.37*</td>
</tr>
<tr>
<td>FB1 (5.0 μM)</td>
<td>5.07±2.12</td>
<td>6.91±2.99*</td>
<td>1.36±0.17*</td>
</tr>
<tr>
<td>Impure FA1 (0.5 μM)</td>
<td>5.91±1.95</td>
<td>7.49±1.29*</td>
<td>1.33±0.27*</td>
</tr>
<tr>
<td>Impure FA1 (5.0 μM)</td>
<td>5.38±0.88</td>
<td>13.05±2.37*</td>
<td>2.47±0.57*</td>
</tr>
<tr>
<td>Purified FA1 (0.5 μM)</td>
<td>4.71±0.56</td>
<td>0.52±0.20</td>
<td>0.11±0.06</td>
</tr>
<tr>
<td>Purified FA1 (5.0 μM)</td>
<td>5.85±0.77</td>
<td>0.95±0.60</td>
<td>0.11±0.02</td>
</tr>
</tbody>
</table>

Values are means±S.D. of triplicate determinations. Values with an asterisk are significantly different ($P < 0.05$) from corresponding control treatment values.

Fig. 3. HPLC–MS profile of impure preparation of N-acetyl fumonisin B1 (impure FA1) showing contaminating peaks with retention times of 12.7 and 13.2 min, and the mass spectrum of the peak eluting at 12.7 min.
and two sodium molecules at m/z 783 and 808, while the two earlier eluting components only showed the protonated molecule at m/z 764 but no sodium adducts. Both of the contaminants were retained on a SCX column as FB1 does which indicated that they had a free amine group whereas the FA1 was not retained. We previously purified a small amount of FA3 from a F. verticillioides strain that made no FBI, but instead produced primarily FB3 and FB4 (Poling and Plattner, 1996). During its purification we observed a small amount of a contaminant that we separated from the FA3. The mass spectra of the FA3 and the contaminant had the same molecular weight, 747, and similarly showed sodium adducts for the FA3, but not for the contaminant. We identified this contaminant as the 3-O-acetyl FB3 because on hydrolysis of the mixture we only obtained one product, HFB3 (S. M. Poling, unpublished). Because FA3 lacks the 5-OH group of FAI, only a single rearrangement product formed.

To examine the conditions favoring rearrangement, an FA1 preparation containing small amounts of the two rearrangement products was heated for 9 h at 95 °C in either 5% acetic acid in 50/50 CH3CN/H2O or 50/50 CH3CN/H2O with no acid added. Rearrangement to O-acetylated products was favored in the acidic media and the amounts of the two products increased. Rearrangement product amounts remained unchanged on heating in the neutral solution when compared to the amounts of the O-acetylated forms initially present. When the contaminated FA1 was dissolved in 5% acetic acid in 10/90 CH3CN/H2O and heated at 95 °C, a stable equilibrium ratio between 3-O-acetyl FB1 and FA1 was reached between 2 and 3 h (Fig. 4) and remained the same for another 4 h. The amount of 5-O-acetyl FB1 increased more slowly and only approached the amount of 3-O-acetyl FB1 after 7 h (Fig. 5). The hydrolysis rate was approximately twice as fast in 5% acetic acid in 10/90 CH3CN/H2O than in 50/50 CH3CN/H2O. In an attempt to isolate 3-O-acetyl FB1, FA1 was heated in 5% acetic acid in 50/50 CH3CN/H2O for 2 h to maximize the amount of 3-O-acetyl FB1, while minimizing the amount of 5-O-acetyl FB1. When the mixture was recovered with a tC18 cartridge and the eluate in 50/50 CH3CN/H2O allowed to stand overnight at room temperature, about 65% of the 3-O-acetyl FB1 rearranged back to FA1, whereas the amount of the 5-O-acetyl form changed very little. When this mixture was separated on a weak anion exchange cartridge using the procedure for separating FB1 from FA1 (Poling and Plattner, 1999), the 3- and 5-O-acetyl FB1 eluted in the fraction in which FB1 would have eluted. If the fraction was allowed to stand at room temperature in the 5% acetic acid eluate for 4 days and resampled, there was no rearrangement back to FA1. The rearrangement of 3-O-acetyl FB1 back to FA1 in the absence of acid was too rapid to continue the isolation. The stability of the O-acetylated form in the presence of less concentrated acetic acid was also observed in the analytical samples.

Fig. 4. HPLC-MS profile of impure FA1 after treatment with 5% acetic acid 10:90 acetonitrile:water at 95°C for 2 h.
that were diluted 50- to 200-fold before analysis and showed little change when reanalyzed.

After SCX column clean-up, the contaminating peaks at 12.7 and 13.2 min were removed, resulting in a purified preparation of FA1 (Fig. 6). Also lost was the ability of the impure preparation of FA1 to inhibit ceramide synthase. Sphinganine content and Sa/So ratios of liver slices treated with purified FA1 were not significantly different from control slices (Table 1). Approximately 1 year after preparation of the purified FA1 the material, which had been stored as a dry powder in a desiccator at room temperature, was again found to be capable of elevating sphinganine levels, and again had similar HPLC-MS profiles as seen in Fig. 3. A similar phenomenon occurred with an FA1 sample stored for about the same period of time as a dry film at 4°C.

![HPLC chromatogram of impure FA1](image1.png)

**Fig. 5.** HPLC chromatogram of impure FA1 after treatment with 5% acetic acid 10:90 acetonitrile: water at 95°C for 7 h.

![HPLC-MS profile](image2.png)

**Fig. 6.** HPLC-MS profile of FA1 purified by SCX chromatography showing FA1 peak with retention time of 16.2 min and the corresponding mass spectrum.
4. Discussion

Substantial evidence has accumulated that the underlying mechanism by which fumonisin B1 and related compounds cause both acute and chronic toxicity is through the disruption of sphingolipid metabolism as a result of the abilities of the toxins to inhibit ceramide synthase (sphingosine- and sphinganine-N-acyl transferase) (Merrill et al., 1996; Riley et al., 1996, 1999; EHC, 2000). Merrill et al. (1996) reported that FA1 had \( <2\% \) of the ceramide synthase inhibition activity of FB1. Thus the primary amino group of the B series of fumonisins is necessary for inhibition of ceramide synthase, and, by presumption, the subsequent disruption of sphingolipid metabolism, followed by overt toxicity, including carcinogenicity (Schroeder et al., 1994). Supporting this hypothesis are the findings of Gelderblom et al. (1993) that N-acetylated FB1 is significantly less cytotoxic than FB1 to rat primary hepatocytes at high doses (1 mM), and that when fed to rats FA1 lacked the cancer-initiating potential of FB1. FA1 was also found to be less phytotoxic than FB1 towards corn seedlings and tomato leaves (Abbas et al., 1993; Lamprecht et al., 1994). It should be noted that hydrolyzed FB1 (aminopentol-1; API), but not FB1 itself, can be a substrate for N-acylation by ceramide synthase (Humpf et al., 1998). The product of this reaction, palmitoyl aminopentol-1, is also an inhibitor of ceramide synthase in cultured colonic cells (Humpf et al., 1998). Whether or not the acetylated form of API is an inhibitor of ceramide synthase is not known.

We first reported in 1997 the necessity to take precautions to ensure the purity of FA1 preparations prior to their use in toxicological or other experimental procedures (Norred et al., 1997). Although at that time the contaminants in our preparation were unknown, they could be readily removed by SCX column clean-up yielding approximately 99% FA1. We speculated that the contaminants were FB1 and possibly other fumonisins. The present report demonstrates that very little, if any, FB1 was contained in the impure FA1, because there was no HPLC peak at the retention time of FB1 (12.56 min under the conditions described in Materials and Methods). There was, however, clear evidence of \( O \)-acylated fumonisin derivatives, and it is plausible that these compounds are responsible for the ceramide synthase inhibition we observed. Other contaminants cannot be entirely ruled out. The results suggest that the contaminants may be more potent inhibitors of ceramide synthase than FB1. The \( O \)-acylated compounds were present in impure FA1 at a level of approximately 20% that of FA1 (as judged by peak height), yet were as potent or even more so as purified FB1 in elevating levels of sphinganine in the liver slices (Table 1). However, it should be pointed out that since the detector response factor of the \( O \)-acylated forms relative to FA1 or FB1 is unknown, the actual quantity of the contaminants in the preparation we used is also unknown.

The results we obtained strongly suggest that FA1, once purified, spontaneously undergoes rearrangement, reaching an equilibrium state between FA1 and the 3-\( O \)-acetyl and 5-\( O \)-acetyl derivatives. This rearrangement occurred in separate preparations, one stored as a powder in a desiccator at room temperature and the other as a dry film in a refrigerator. Each of the preparations was in storage for about 1 year, and at present it is unknown how long a period of time is required for the equilibrium condition to occur. The rearrangement of FA1 and the two putative \( O \)-acetylated derivatives depends on the acidity of the media. That there is no formation of FB1 favors the interpretation of the mechanism as intramolecular. The formation of a single rearrangement product for FA3, whereas FA1 yields two rearrangement products, is in agreement with the involvement of the 3- and 5-OH groups in the rearrangement and indicates that the 10-OH group is not involved. While the exact nature of the rearrangement is unknown, we speculate the intramolecular 1–5 and 1–7 shifts are the most likely mechanism. The equilibrium condition is also \( p \text{H} \) dependent, with the formation of the \( O \)-acetylated forms being favored under acidic conditions (Figs 4 and 5). The rearrangement products (putative 3- and 5-\( O \)-acetyl FB1) behave on SCX, weak anion exchange and C18 columns as if the amino group is not acetylated. The mass spectra also indicate the rearrangement products have a non-acetylated amino group. We believe this shows the impurities that form on standing to be 3- and 5-\( O \)-acetyl FB1. Even if these are not the structures of the impurities, the impurities definitely have free amine groups and it is therefore reasonable to assume that they are responsible for the biological activity seen in FA1 samples that are not purified immediately prior to testing.

Van der Westhuizen et al. (1998) investigated the ability of various structural analogs of fumonisin B1, including FA1, to inhibit sphingolipid biosynthesis in rat primary hepatocytes. FA1 inhibited ceramide synthase to the same extent as FB1, and the authors concluded that the presence of a primary amino group is not required for inhibition. Although cytotoxicity of the FA1 preparation used by Van der Westhuizen et al. (1998) was not determined, the authors concluded that since earlier toxicity studies (Gelderblom et al., 1993) showed that FA1 was less toxic to hepatocytes than FB1, then the structural requirements of fumonisins for ceramide synthase inhibition and for cytotoxicity must be different. Based on our experiences reported herein regarding the instability of FA1, it is conceivable that the FA1 preparation used in the experiments of Van der Westhuizen et al. (1998) had undergone rearrangement to \( O \)-acetylated derivatives capable of inhibition of
ceramide synthase. Although purity of their fumonisin preparations was determined by $^{13}$C-NMR, HPLC and TLC, it is not stated whether the purity was assessed immediately prior to the experiments, or only at the time the compounds were purified. It would be of interest to analyze their FA1 preparation by HPLC-MS to determine whether O-acetylated derivatives are present.

In conclusion, the results we obtained show that purified FA1 free of O-acetyl rearrangement products has no or very low activity as an inhibitor of ceramide synthase. Coupled with the findings of several other laboratories that FA1 is less toxic than FBI and other fumonisin analogs, our findings in this study are consistent with the hypothesis that inhibition of ceramide synthase and the resulting disruption of sphingolipid metabolism are the underlying mechanism for the biological activity of fumonisin mycotoxins.

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References


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