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Modified Hydra Bioassay to Evaluate the Toxicity of Multiple Mycotoxins and Predict the Detoxification Efficacy of a Clay-Based Sorbent

KA Brown, T Mays, A Romoser, A Marroquin-Cardona, NJ Mitchell, SE Elmore, and TD Phillips*

Veterinary Integrative Biosciences Department, College of Veterinary Medicine, Texas A&M University, College Station, TX

Abstract

Food shortages and lack of food supply regulation in developing countries often leads to chronic exposure of vulnerable populations to hazardous mixtures of mycotoxins, including aflatoxin B₁ (AFB₁) and fumonisin B₁ (FB₁). A refined calcium montmorillonite clay (i.e. UPSN) has been reported to tightly bind these toxins, thereby decreasing bioavailability in humans and animals. Hence, our objectives in the present work were to examine the ability of UPSN to bind mixtures of AFB₁ and FB₁ at gastrointestinally relevant pH *in vitro*, and to utilize a rapid *in vivo* bioassay to evaluate AFB₁ and FB₁ toxicity and UPSN efficacy. Isothermal sorption data indicated tight AFB₁ binding to UPSN surfaces at both pH 2.0 and 6.5, but substantially more FB₁ bound at pH 2.0 than 6.5. Site-specific competition occurred between the toxins when exposed to UPSN in combination. Importantly, treatment with UPSN resulted in significant protection to mycotoxin-exposed hydra maintained at pH 6.9–7.0. Hydra were exposed to FB₁, AFB₁ and FB₁/AFB₁ combinations with and without UPSN. Toxic response over 92 hours was rated based on morphology and mortality. Hydra assay results indicated a minimum effective concentration (MEC) of 20 µg/mL for AFB₁, while the MEC for FB₁ was not reached. The MEC for co-exposure was 400 µg/mL FB₁ + 10 µg/mL AFB₁. This study demonstrates that UPSN sorbs both mycotoxins tightly at physiologically relevant pH levels, resulting in decreased bioavailability, and that a modified hydra bioassay can be used as an initial screen *in vivo* to predict efficacy of toxin binding agents.

Keywords

aflatoxin; fumonisin; NDA; HPLC; hydra toxicity; clay adsorption

Introduction

Aflatoxin B₁ (AFB₁) and Fumonisin B₁ (FB₁) frequently co-contaminate foods such as maize and grain products in developing countries where concentrations frequently exceed U.S. action limits (Kimanya et al. 2008; Kpodo et al. 2000; Prathap Kumar et al. 1997). AFB₁ is a mycotoxin produced by *Aspergillus flavus* and *A. parasiticus* fungi. AFB₁ (Fig. 1A) is known to be hepatotoxic and immunotoxic (Bianco et al. 2012), as well as cytotoxic (Hochstenbach et al. 2010) and antinutritional (Osborne et al. 1982). AFB₁ is currently classified as a carcinogen by the International Agency for Research on Cancer (IARC 2002) and has been implicated in liver cancer associated with consumption of contaminated food (Qian et al. 1994; Ross et al. 1992; Wang et al. 1996; Yu et al. 1997) and hepatic failure

*Corresponding author: T. Phillips; Tel.: +1-979-845-6414; fax: +1-979-862-4929 tphilips@cvm.tamu.edu.

resulting in death (Lewis et al. 2005). Furthermore, AFB₁ has been associated with hepatocellular carcinoma (HCC) in humans (Peers and Linsell 1973; Peers et al. 1976). Fumonisin B₁ (Fig. 1B) is also hepatotoxic and is produced by *Fusarium verticillioides* fungi (WHO 2001). Whereas AFB₁ is classified as a group 1 carcinogen due to sufficient evidence indicating its ability to initiate cancer, FB₁ is classified as a group 2B carcinogen (IARC 1993, 2002) due to its possible carcinogenicity to humans. It has been suggested that FB₁ acts to promote the toxic effects of AFB₁ when ingested together (Gelderblom et al. 2002; McKean et al. 2006). In rainbow trout, the FB₁ promotional activity in AFB₁-initiated liver tumors was correlated with modulated sphingolipid metabolism (Carlson et al. 2001). FB₁, which is structurally similar to sphingoid bases, has been shown to block sphingolipid synthesis (Riley et al. 2001; Wang et al. 1991), which consequently alters cellular signaling and function (Riley et al. 1998), including membrane structure integrity (Wu et al. 1995). These cellular disruptions could result in increased susceptibility to other toxins, such as AFB₁.

In developing countries, the health consequences of mycotoxin ingestion are frequently enhanced in humans and animals. Although it is known that normal cooking processes do not effectively degrade AFB₁, certain processing techniques can reduce FB₁ toxicity. For example, nixtimalization (alkaline processing) and twin-screw extrusion can reduce bioavailability and toxicity of FB₁ (Burns et al. 2008; Jackson et al. 2011; Voss et al. 2011). The extrusion method utilized by Voss et al. (2011) was able to protect against FB₁-induced nephrotoxicity and disrupted sphingolipid metabolism in rats. Nixtimalization does produce a hydrolyzed fumonisin byproduct, but it has been shown to be much less toxic than its parent compound (Voss et al. 2009). However, due to the fact that these techniques to reduce toxicity are not ubiquitously employed, implementation of enterosorbent intervention strategies is an attractive option.

Geophagy, the intentional ingestion of clay, is known to occur in many species, including humans, and has been shown to produce overall health benefits (Carretero 2002). It is thought that the consumption of clays by animals and birds may occur innately as a mechanism of dietary supplementation and/or toxin mitigation (Aufreiter 2001; Gilardi et al. 1999). Historically, humans have practiced geophagy to acquire nutritional supplementation and to aid in the relief of gastrointestinal upset (Diamond 1999; Johns and Duquette 1991). Uniform particle size NovaSil (UPSN) is a refined calcium montmorillonite clay which has been shown to sorb AFB₁ and FB₁ *in vitro* and *in vivo*, thereby decreasing toxin bioavailability and systemic exposure (Phillips 1999; Phillips et al. 2002; Robinson et al. 2012; Wang et al. 2008). Mycotoxin sorption onto surfaces of UPSN likely occurs by way of an electron donor acceptor mechanism, whereby positively charged ions are attracted to negatively charged, isomorphically substituted platelets of the clay (Phillips et al. 2008). Chemicals with areas of electron deficiencies can also be attracted to the platelets (Haderlein et al. 1996). Although AFB₁ and FB₁ bind tightly to parent NovaSil clay, no adverse effects on vitamin and mineral utilization were observed in clinical trials in Africa (Afriyie-Gyawu et al. 2008; Afriyie-Gyawu et al. 2005; Wang et al. 2005). Also, levels of dioxin/furans, as well as metals analysis for UPSN were previously published by our laboratory, indicating concentrations well below the provisional tolerable daily intake (Marroquin-Cardona et al. 2011).

Hydra vulgaris can serve as an initial screening tool and a predictive model for the efficacy of potential mycotoxin enterosorbents *in vivo*. Hydra are freshwater polyps, which are three cell layers thick and highly sensitive to environmental changes and diverse chemicals. Adult hydra have previously been utilized in our laboratory to determine the dosimetry and minimal effective concentrations of various chemicals, including mycotoxins and environmental pollutants that result in toxicity (and mortality) (Afriyie-Gyawu et al. 2005;

Huebner et al. 2000; Pascoe et al. 2002; Yang et al. 1993). Hence, our objectives in this study were to examine binding efficacy of UPSN to AFB₁, FB₁, and AFB₁+FB₁ at pH levels relevant to the gastrointestinal tract of humans (i.e., 2.0 and 6.5), and to apply a modified hydra bioassay to confirm sorption and protection *in vivo* against both mycotoxins in the presence of UPSN.

Materials and Methods

Reagents

High Pressure Liquid Chromatography (HPLC) grade methanol and acetonitrile reagents, as well as reagent grade glacial acetic acid, sodium hydroxide, sodium borate, sodium cyanide, dibasic potassium phosphate, naphthalene-2,3-dicarboxaldehyde (NDA), and pH buffers (4.0, 7.0 and 10.0) were purchased from VWR (Atlanta, GA). AFB₁ and FB₁ were purchased from Sigma Aldrich (Saint Louis, MO). UPSN was obtained from Texas Enterosorbents (Bastrop, TX). Ultrapure deionized water (18.2 MΩ) was generated within the lab using an Elga™ automated filtration system (Woodridge, IL).

HPLC analysis of AFB₁

AFB₁ was quantified by reversed-phase HPLC. The HPLC system consisted of a fluorescence detector (Waters 2475), a photochemical reactor for enhanced detection (PHRED-12) (Aura Industries, Inc., New York, NY), a binary pump (Waters 1525), and an autosampler (Waters 717). A reversed-phase Spherisorb® C18 column with a 5 μm particle size (4.6×150mm, Waters) was used to separate AFB₁ from the solution. Excitation and emission wavelengths were set at 360 and 420 nm, respectively. Quantification was achieved by peak-area measurement using Breeze™ HPLC analysis software (Waters). Appropriate standards were prepared for each isothermal run. Specifically, an AFB₁ standard calibration curve was generated by utilizing a serial dilution from 0.1 to 10 μg/mL. Standards and samples (100 μL) were loaded onto the autosampler tray for analysis. The mobile phase consisted of 57:41:2 acetonitrile:methanol:water with a flow rate of 1.0 mL/min. The run time was set at 19 min with an injection volume of 10 μL. Under the conditions of this study, AFB₁ exhibited a retention time of 16.7 min. AFB₁ correlation coefficients for all curves were 0.9959.

Derivatization of FB₁ by NDA

Due to the lack of a fluorescent component on the molecule of FB₁, derivatization is necessary for HPLC detection. The derivatization method of FB₁ followed procedures previously reported (Bennett and Richard 1994). Sorption assays were accomplished using HPLC analysis with naphthalene-2,3-dicarboxaldehyde (NDA) derivatizing agent as the detection method for FB₁. NDA provides a stable derivative that resists decay over time, allows for faster sample throughput and also allows for the re-analysis of a sample without repetition of the derivatization step (Office of the Texas State Chemist 2008). Briefly, 1 mL of sample extract (containing FB₁) was placed into a 40 mL amber vial. Sodium borate buffer (1 mL, 0.05M) was added to the vial, followed by 0.5 mL sodium cyanide (0.13 mg/mL) and 0.5 mL NDA (0.25 mg/mL). The vials were capped, vortexed and placed in a water bath (60° C) for 20 min, followed by refrigeration (4° C) for 4 min. Next, 7 mL of 60:40 acetonitrile:phosphate buffer was added to each vial. The vials were then vortexed and 1.5 mL was syringe filtered into a 2 mL amber HPLC vial for FB₁ analysis. A FB₁ standard was diluted with purified water to form the following concentrations: 0.1, 0.5, 1.0, 2.5, 4.0, 5.5, 7.0, 8.5, 10.0, 25.0, 50.0, 80.0, 100.0, and 125.0 μg/mL. Each of these concentrations was then derivatized in the same manner as the sample extracts.

Chromatographic analysis of FB₁

FB₁ was quantified by reversed-phase HPLC using a reversed-phase μ Bondapak™ C18 analytical column with a 10 μ m particle size (3.9×150mm, Waters). Excitation and emission wavelengths were set at 420 and 500 nm, respectively. Calibration curves for FB₁ samples consisted of nine points per isotherm, ranging from 0.1 to 10.0 ppm and 2.5 to 125.0 μ g/mL. A volume of 100 μ L (standards and samples) was loaded onto the autosampler tray for analysis. The mobile phase consisted of 57:42:1 acetonitrile:water:acetic acid with a flow rate of 1.0 mL/min. The run time was set at 8 min with an injection volume of 10 μ L. FB₁ exhibited a retention time of approximately 5.8 min for low concentration and combination analyses and 4.7 min for high concentration analyses. FB₁ calibration curves yielded correlation coefficients of 0.9939.

In vitro sorption assay for AFB₁ and FB₁

In this study, we assessed sorption of AFB₁ and FB₁ to UPSN and the ability of UPSN to prevent toxicity at pH levels of 2.0 and 6.5. Sorption assays for both mycotoxins were performed in purified deionized water at pH 2.0 and 6.5. The pH of water was adjusted with 1M HCl or 0.1M NaOH. AFB₁ and FB₁ aqueous solutions (8 μ g/mL) were prepared individually at both pH levels. Previous results from individual isotherms performed in our laboratory indicated that at pH 6.5, NS has a low affinity for FB₁; therefore the combination isotherm assay was conducted at pH 2.0 only. AFB₁ (2.5 mg/mL in acetonitrile) and FB₁ (2.5 mg/mL in 1:1 acetonitrile:water) (Sigma Aldrich) were diluted with purified deionized water until a concentration of 8 μ g/mL was achieved. The concentration of AFB₁ was verified using a UV-Vis spectrophotometer (UV-1800, Shimadzu, Columbia, MD) at 362 nm and the concentration of FB₁ was verified by HPLC (Waters 2475, Milford, MA) using methods previously described.

Isotherms were performed using a modified version of the Grant and Phillips method (Grant and Phillips 1998). Briefly, a clay suspension (2 mg/mL) was prepared by placing 100 mg of UPSN clay into a 50 mL Erlenmeyer flask. Then, 50 mL of purified water was added and the suspension was stirred continuously during sample preparation. For individual mycotoxin isothermal analysis, 11 different concentrations ranging from 0.4 to 8.0 μ g/mL AFB₁ or FB₁ were prepared. The combination isotherm consisted of three (0.4, 4.0 and 8.0 μ g/mL) concentrations. Each concentration was assessed in triplicate. Appropriate amounts of purified water and AFB₁ and/or FB₁ were added to the sample tubes to achieve the desired concentrations, yielding a total sample volume of 5 mL. Three control tubes were also prepared, which consisted of water without UPSN, water with UPSN, and 8.0 μ g/mL AFB₁ or FB₁. A 50 μ L volume of a 2 mg/mL UPSN suspension (0.02%) was added to each of the sample tubes and the control tube labeled “water with UPSN”. All test tubes were capped, shaken for 2 hr at 1,000 rpm, and centrifuged at 2,000 rpm for 20 min. A 1.5 mL volume of supernatant was filtered by syringe (0.45 μ m) into a 2 mL amber HPLC vial for AFB₁ HPLC analysis. FB₁ isotherms were performed in the same manner as for AFB₁. To further evaluate UPSN-FB₁ binding potential at pH 6.5 an additional isotherm was performed. Eleven concentrations between 5.0 and 100.0 μ g/mL FB₁ were assessed in the presence of 2% UPSN. UPSN was measured out by weight (100.0 mg per 5 mL sample) and the experiment proceeded as previously stated for UPSN isotherms. Sorption data was analyzed using TableCurve 2D software.

A similar procedure was followed in a separate experiment to examine AFB₁ and FB₁ binding site locations on the UPSN by heat collapsing the interlayer and comparing binding capacity to intact UPSN. Collapse was achieved by heating UPSN to 200° C for 30 min, then increasing the heat to 800° C for 1 hr, as described in Grant and Phillips, (1998) and Robinson et al., (2012). Collapsed UPSN was then added to samples containing 400 ppm

FB₁, 10 ppm AFB₁ and their combination (as described for the isotherm analyses with intact UPSN). The concentrations were further verified by measuring the absorbance of AFB₁ at 362 nm on a scanning UV visible spectrophotometer (Shimadzu, Model 1601PC) and by measuring FB₁ content via HPLC. Samples were analyzed for FB₁ content in the supernatant, as previously stated. Collapsed UPSN isothermal experiments were performed at pH 6.5.

Hydra bioassays

Hydra vulgaris were obtained from Environment Canada (Montreal, Qc) and maintained at 18°C. The Wilby classification method (Wilby 1988) was used with modification to rate toxicity based on the morphological condition of the hydra. An illustration of the classification system utilized is included in Figure 2. The judging method of hydra morphology used in this bioassay is objective and is based on pre-defined states of morphology which have been represented in detail in the previous literature. The incremental grading scale used for this assay is very descriptive and the accuracy of this method has been validated in our laboratory with successful replication. The assay modification in this work included more extensive and quantitative parameters of toxicity, as well as intermediate levels of toxicity ratings and increased length of exposures. Specifically, this method included monitoring times at shorter intervals during the first two days (0, 4, 20, and 28 hr) followed by monitoring at 24 hr intervals for three days (44, 68, and 92 hr). Solutions were not changed during testing. Hydra biological response was investigated after exposures to AFB₁ and FB₁ separately and in combination, as well as with and without UPSN. Mature, non-budding hydra were fasted 24 hr prior to testing and during 92 hr testing periods. Hydra of similar size and shape were chosen for testing in order to minimize disparities between samples. Controls for this experiment included hydra media (consisting of 18.2 MΩ water, 4 mg/L EDTA, 115 mg/L N-tris[Hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES), 147 mg/L CaCl₂ adjusted to pH 6.9-7.0), treated controls for acetonitrile (99.9% HPLC grade), and UPSN (0.01%, 0.7%, 1.4 %, and 2%). Acetonitrile control groups were included during all AFB₁ testing periods since this solvent was used as a vehicle for mycotoxin stock solutions. UPSN inclusion percentages were based on values previously utilized in animal and human studies (Marroquin-Cardona et al. 2011; Robinson et al. 2012; Wang et al. 2008). Toxin treatment groups included AFB₁ (10, 20, or 30 µg/mL) and FB₁ (100, 200, 300, or 400 µg/mL). From the scientific literature, dietary FB₁ exposures rarely exceed concentration levels above 400 µg/mL, therefore higher concentrations were not tested in this study (Kpodo et al. 2000; Prathapkumar et al. 1997). Test solutions including UPSN were prepared by shaking at 1000 rpm for 2 hr and centrifuging for 20 min at 2000 rpm prior to pouring the supernatant directly into the Pyrex dishes.

To test AFB₁ and FB₁ co-exposure toxicities, a series of mixtures was prepared containing 5-20 µg/mL AFB₁ and 100-400 µg/mL FB₁ to determine minimum effective concentration (MEC) for the combination. The MEC was based on the lowest dose that caused 100% mortality at 92 hr. Specifically, 5, 10, 15, and 20 µg/mL AFB₁ were combined with 400 µg/mL FB₁ and 100, 200, and 300 µg/mL FB₁ were combined with 20 µg/mL AFB₁. AFB₁ and FB₁ combinations were prepared in hydra media with or without UPSN. Samples were incubated at 18° C for 24 hr before exposing the hydra. Toxicity following exposure to 400 µg/mL FB₁ + 10 µg/mL AFB₁ was examined in the presence of UPSN at concentrations equal to 0.01 %, 0.7 %, and 1.4 %. These test solutions were shaken for 2 hr, then centrifuged (2000 rpm) for 20 min prior to incubation (18° C) for 20 min. Solutions were placed in Pyrex dishes immediately before introducing the hydra. For each sample, three hydra were introduced into 4 mL of test media and kept at 18° C. For all hydra experiments the average toxicity rating was determined by calculating the average score for morphological changes for a specific toxin concentration at a specific time point.

Significance was determined by comparing the morphological condition scores of the treated group with those of the untreated controls.

Statistical Analysis

An ANOVA with post-hoc comparison was employed to analyze statistical significance for the high concentration FB₁ isotherm, competition binding assay, heat-collapse experiment, as well as to calculate significant change in hydra morphological response for each particular treatment scenario, as compared to untreated control hydra. All experiments analyzed were run in triplicate. Results were considered significant at $p < 0.05$.

Results

Isothermal analysis

Sorption of AFB₁ and FB₁ was evaluated by fitting the data points collected for each experimental analysis using the Langmuir (Langmuir 1916) or Freundlich (Freundlich 1909). The r^2 for the isothermal graphs at pH 2 were 0.96 and 0.91 for AFB₁ and FB₁ respectively. The r^2 for the graphs at pH 6.5 were 0.93 and 0.62 for AFB₁ and FB₁ respectively. Parameters of $Q_{\max}$ and K_d were estimated at each pH level with low concentrations of toxin and UPSN (Figure 3). The $Q_{\max}$ values for AFB₁ were 0.43 mol/Kg and 0.25 mol/Kg at pH 2.0 and 6.5, respectively (Figure 3a). The $Q_{\max}$ value for FB₁ at pH 2.0 was 0.10 mol/Kg (Figure 3b). At pH 6.5, UPSN exhibited a low affinity for FB₁ and the resulting isothermal data did not fit the Langmuir model even at a higher concentration of clay. However, at the higher concentration of clay, a significant increase in FB₁ sorption was observed at all tested concentrations (Figure 4). FB₁ in solution was reduced by nearly 77% in the 100 $\mu\text{g/mL}$ sample when treated with UPSN. When AFB₁ and FB₁ were combined, and the reaction was initiated in the tube with UPSN, a decreased percentage of binding to UPSN was observed for both mycotoxins (Figure 5a). More specifically, UPSN bound approximately 5% less AFB₁ and 16% less FB₁ when both toxins were present at the same level (8 $\mu\text{g/mL}$). In UPSN that had been heat-collapsed, FB₁ binding decreased significantly as compared to intact UPSN (Figure 5b), indicating interaction of FB₁ within the interlayer.

Hydra bioassay

Hydra response to AFB₁ exposure resulted in an MEC of 20 $\mu\text{g/mL}$ (Figure 6a), which was evidenced by a marked and consistent decrease in physiologic condition and 100% mortality (0 on the Wilby scale) by 92 hr. At all time-points, (with the exception of 0 hr) 10-30 $\mu\text{g/mL}$ AFB₁ treated samples were significantly different from controls ($p < 0.05$). UPSN clearly conferred protection to the hydra, resulting in toxicity ratings with no significant difference from controls (Figure 6b). ACN treatment groups were not significantly different from media controls at any time point (data not shown). The MEC for FB₁ was not reached in this study (Figure 6c); however, severe gross morphological changes were observed at 400 $\mu\text{g/mL}$ FB₁ (average toxicity rating, 4.2). Concentrations of 300 and 400 $\mu\text{g/mL}$ FB₁ resulted in significant toxicity between the 20-92 hr time points. FB₁ concentration of 200 $\mu\text{g/mL}$ produced significant toxicity at the 44 and 92 hr time points, whereas 100 $\mu\text{g/mL}$ produced significant toxicity at 92 hr only. At every time point, 2 % UPSN conferred protection against the toxic effects of FB₁ (Figure 6d). The MEC of AFB₁ and FB₁ combined was determined to be 400 $\mu\text{g/mL}$ FB₁ + 10 $\mu\text{g/mL}$ AFB₁ (Figure 6e). The combination of AFB₁ + FB₁ was more toxic than either individual mycotoxin. A statistically significant dose response relationship was demonstrated with addition of UPSN (Figure 6f). The 0.01 % UPSN + 400 $\mu\text{g/mL}$ FB₁ + 10 $\mu\text{g/mL}$ AFB₁ group was significantly different from controls at 4, 28, 68, and 92 hr, while the 0.7 % UPSN group was significantly different at the 68-92 hr time points only. The highest level of UPSN inclusion (1.4 %) was not significantly different from controls at any time point. Results indicate that UPSN significantly protected

hydra from the toxic effects of AFB₁ and FB₁ individually and combined throughout the 92 hr assay duration at each concentration tested. Even at the lowest levels of UPSN inclusion, toxic effects were decreased. The prevention of toxicity to the adult hydra in the presence of UPSN is demonstrated in Figure 7, which illustrates the effects of 10 µg/mL AFB₁, 400 µg/mL FB₁, and 10 µg/mL AFB₁ + 400 µg/mL FB₁ at 44 hr exposure, with and without the presence of 1.4 % UPSN. UPSN prevented the toxic effects of AFB₁ and FB₁, both singly and in combination. The images of UPSN-protected hydra reveal an additional morphological feature known as “budding” (asexual reproduction), which is an indicator of a healthy morphologic state.

Discussion

At physiologically relevant pH levels commonly found in the stomach and intestine (pH 2.0 and pH 6.5, respectively), AFB₁ was bound tightly to UPSN, a finding similar to our previous laboratory results with parent NovaSil clay. In previous work with NovaSil clay we have demonstrated that this binding is thermodynamically favored (Pimpukdee et al. 2004) and may be attributed to the chemisorption of aflatoxins at surfaces within the internal (interlayer) regions on the clay; whereas, the exterior surfaces of the clay were responsible for only minor sorption of aflatoxins. Importantly, a good correlation was observed between the magnitude of positive charge on carbons C₁₁ and C₁ of the β-dicarbonyl system in aflatoxins and the strength of sorption of aflatoxin congeners and similar ligands. These findings suggested both stereo- and regio-selectivity for the interaction of aflatoxins onto the surfaces of calcium montmorillonite clay. (Grant and Phillips 1998; Phillips et al. 2002; Phillips et al. 1988). Unlike AFB₁, the interaction of FB₁ with clay surfaces indicated binding at pH 2.0, but a relatively low affinity of binding at pH 6.5 *in vitro*. The FB₁ molecule is amphoteric with an amine located at the C₂ position which becomes protonated (and positively charged) at low pH values. This enhances its ability to undergo cation exchange at negatively charged interlayer surfaces of montmorillonite clay (Phillips et al. 2008). Also, since FB₁ is bulkier than AFB₁, it may occupy fewer internal binding sites due to steric hindrance and/or decreased affinity to UPSN. Importantly, due to the larger molecular weight of FB₁ (721.83 g/mol) versus AFB₁ (312.3 g/mol), on a molar basis, there would be fewer molecules of FB₁ in competition with AFB₁ for available sites which may explain less binding of FB₁ in combination with AFB₁.

Our laboratory routinely performs equilibrium isotherms using sorption models to characterize surface chemistry and the affinity and binding capacity of diverse chemicals. In earlier work with aflatoxin and fumonisin sorption onto the surfaces of parent NovaSil clay, we have typically observed a good fit of the data using the Langmuir model. However, with this clay there is always a slight change in slope of the curve prior to saturation which may indicate the presence of additional binding sites that affect the saturation process. The high *r*² values obtained in this study for AFB₁ (at both pH conditions) and FB₁ (at pH 2) suggest a good fit for the Langmuir model. Moreover, our isothermal data indicates that surfaces on the clay contain saturable sites for the sorption of both toxins.

Previous research in our laboratory with heat-collapsed montmorillonite clay suggests that AFB₁ and FB₁ binding occurs largely within the internal interlayer regions (Marroquin-Cardona 2011; Robinson 2010). This mechanism may act to sequester the mycotoxin(s) in the stomach and reduce dissociation from the clay as pH increases in the intestine in mammals. Heat-collapse of montmorillonite clay causes the layers to dehydroxylate as the temperature nears 800° C, thereby decreasing internal clay interactions and interlayer binding. Marroquin-Cardona (2011) found significantly reduced binding capacity of AFB₁ (as much as 85%) upon heat-collapse of interlayer surfaces in parent NovaSil clay (HSCAS), confirming the previous findings of Grant and Phillips (1998). Also, Robinson

(2010) showed that isothermal analysis of FB₁ binding to collapsed calcium montmorillonite clay did not fit the Langmuir curve at pH 2 suggesting that FB₁, like aflatoxin, needs internal binding sites for maximal sorption. FB₁ binding in the current study decreased significantly with collapsed UPSN as compared to intact UPSN, indicating partial interlayer binding. This is preliminary evidence that both mycotoxins may compete for the same binding sites on UPSN; however, the thermodynamics and kinetics of this interaction will require further investigation using equimolar and radiolabeled toxin concentrations in order to determine the enthalpy of sorption, contribution of each toxin, site affinities and maximum capacities for the combination.

The clay's affinity for the functional groups on AFB₁ and FB₁ would suggest that other chemicals containing these groups may also sorb to the surface of UPSN. However, *in vivo* studies have not supported this general conclusion, suggesting that other factors such as plateau surface density, log P, capacity, affinity and enthalpy of sorption to clay surfaces may also play an important role in selectivity (Grant and Phillips 1998; Phillips 1999). For example, numerous studies in animals and humans following the ingestion of NovaSil clays have shown a lack of interaction with other mycotoxins (e.g. zearalenone, T-2 toxin, DON, cyclopiazonic acid, and ochratoxin A), important vitamins and nutrients, diverse environmental chemicals and organic matter (Afriyie-Gwayu et al. 2008; Afriyie-Gyawu et al. 2005; Grant and Phillips 1998; Phillips et al. 2002; Phillips et al. 2008; Wang et al. 2005). Furthermore, a recent NS study resulted in no detrimental health effects in rats and humans (Robinson et al. 2012).

Although isothermal data indicated differential binding ability at pH 2.0 and 6.5, significant reduction in bioavailability of AFB₁ and FB₁ occurred *in vivo*. Hydra assays revealed efficient UPSN binding of AFB₁ and FB₁ in physiological media, evidenced by the absence of a toxicological effect observed with UPSN treatments. UPSN conferred protection when hydra were exposed to AFB₁ or FB₁ or a combination of the two. At 400 µg/mL, FB₁ was capable of producing substantial toxic effects in the adult hydra; however, a significant decrease in toxicity was observed with 2% UPSN inclusion. Even at pH 6.95, there was significant protection of hydra from FB₁. Similar findings of *in vivo* protection were published by Robinson et al, who reported that NS significantly reduced urinary levels of FB₁ in rats and humans (Robinson et al. 2012).

Hydra assay modifications included in this work allowed for more gastrointestinally relevant conditions. Upon AFB₁ and FB₁ exposure to UPSN, binding occurred and hydra were protected from toxicity for an extended time period. In the gastrointestinal tract, it is necessary for AFB₁ and FB₁ to be bound to an adsorptive agent long enough to be excreted; a process that is typically completed between 24 and 48 hr after ingestion. The literature has shown that there is much variability among individuals and between sexes in gastrointestinal transit times (Degen and Phillips 1996). Closer monitoring at this critical period in hydra reveals that AFB₁ and FB₁ absorption through the lumen can be avoided via a clay-based reduction in bioavailability during normal human and animal digestion (Phillips et al. 2008; Wang et al. 2008). Extended monitoring to 92 hr ensures that toxicity can be prevented at longer time points as well. Thus, this *in vivo* bioassay will allow for physiologically relevant screening of toxicants in a small, but relatively sophisticated organism. A recent paper further elucidated the complexity of this species by identifying a highly conserved gene, *HvCatalase*, responsible for oxidative stress-driven translation of catalase, a protective protein common in higher organisms (Dash and Phillips 2012). The work of Dash and Phillips (2012) demonstrates that hydra possess more complex response mechanisms than once thought. Since mycotoxins are known to exert an oxidative stress response in mammals, oxidative stress-responsive hydra will serve as an appropriate and sensitive model

for mycotoxin exposures, as well as a predictive model for the efficacy of potential mycotoxin enterosorbents *in vivo*.

This research confirms the ability of UPSN, a novel detoxification material, to sorb both AFB₁ and FB₁ and indicates that a modified hydra bioassay can be used to predict toxicity of AFB₁, FB₁ and possibly other mycotoxins or mycotoxin mixtures. *In vitro* sorption results indicated that data from low concentration isotherms may not be predictive of higher dosing concentration binding efficacy *in vivo*. Therefore, in cases of decreased toxin binding affinity, it may be necessary to perform additional analyses (i.e., the high concentration isotherms) and predictive bioassays, such as the hydra bioassay, in order to gain a more accurate understanding of expected animal model and human trial outcomes. Furthermore, results from *in vitro* assays must be utilized carefully and moderately when acting as precursory research for *in vivo* work. Future directions include investigating the hydra mechanism of response to various toxicants and application of the model in testing the efficacy of sorbents for other mycotoxins. Furthermore, knowledge gained regarding HPLC analysis of mycotoxin-surface interactions can be utilized to examine other surface binding interactions of clays and to potentially predict affinity, capacity, and plateau surface density characteristics.

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Citations

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A. Aflatoxin B₁ B. Fumonisin B₁

Figure 1.
2D structures of AFB₁ (A) and FB₁ (B).

Figure 2.

Representative endpoints for the Hydra bioassay, as graded by the Wilby 1988 method. Morphology scale is based on a 10-0 point scale where 10 is a normal, healthy hydra and 0 is a dead, disintegrated hydra. Physiologic condition of the hydra was assessed with a dissecting microscope.

Figure 3.

Langmuir plots of AFB₁ (A) and FB₁ (B) isotherms modeling the observed and predicted Q_{max} values at pH 2.0 and 6.5. The Q_{max} value for AFB₁ at pH 2.0 indicated tight binding, whereas the Q_{max} value for FB₁ indicated binding, although at a decreased affinity. At pH 6.5, the AFB₁ Q_{max} value was lower compared to values obtained at pH 2.0. FB₁ isotherm values did not fit the Langmuir model at pH 6.5, indicating decreased binding.

Figure 4.

FB₁ isothermal analysis after treatment with 2% UPSN. Compared to samples without UPSN, the amount of FB₁ left in solution decreased significantly in all but the 20 µg/mL concentration samples. The darker FB₁ series on the left represent standards without UPSN inclusion; whereas the lighter series to the right shows the FB₁ left in solution after binding to UPSN. Statistical significance is denoted by *(p<0.05).

Figure 5.

Percent individual mycotoxin left in solution after treatment with 0.02% UPSN at concentration levels of 0.4, 4.0, and 8.0 µg/mL AFB₁ and FB₁ (A). Bars labeled 'AFB₁' or 'AFB₁ + FB₁' indicate percent AFB₁ unbound when alone or in combination; whereas bars labeled 'FB₁' or 'FB₁ + AFB₁' indicate percent FB₁ unbound when alone or in combination. This data suggests competition for binding sites, which was more evident at higher toxin concentrations. At all levels, significantly more AFB₁ was left in solution when in combination with FB₁; however, unbound FB₁ was only significantly increased at the highest combination level. Measurements of FB₁ left in solution after binding to 1.4% intact and collapsed UPSN (B), were evidence of FB₁ sorption and the necessity of the interlayer for substantial interaction. Statistical significance is denoted by *(p<0.05).

Figure 6.

Hydra toxicity and protection by UPSN from 0-92 hrs, as measured with the Wilby method. Response to 10-30 $\mu\text{g/mL}$ AFB₁(A); UPSN control (2%) and UPSN-AFB₁ combinations (B); Response to 100-400 $\mu\text{g/mL}$ FB₁(C); UPSN control (2%), and UPSN-FB₁ combinations (D); Response to MEC concentrations of 10 $\mu\text{g/mL}$ AFB₁, 400 $\mu\text{g/mL}$ FB₁, and 10 $\mu\text{g/mL}$ AFB₁ + 400 $\mu\text{g/mL}$ FB₁(E); and UPSN (0.01%, 0.7%, and 1.4%) protection (F). UPSN was able to protect the hydra at all time-points from the toxic effects of AFB₁ and FB₁. In the combination toxicity, UPSN dose-dependently reduced toxic effects of AFB₁ and FB₁. Media controls were included for comparison. Statistical significance is denoted by *($p < 0.05$).

Figure 7.

Hydra morphology in response to 10 $\mu\text{g/mL}$ AFB₁, 400 $\mu\text{g/mL}$ FB₁, and 10 $\mu\text{g/mL}$ AFB₁ + 400 $\mu\text{g/mL}$ FB₁ (left) at 44 hrs. An additional group of hydra treated identically to those pictured in the left-hand column, but in the presence of 1.4 % UPSN (right column) are protected from mycotoxin exposures. The occurrence of asexual reproduction (budding) in hydra that have been protected with UPSN is noteworthy and indicative of a healthy morphologic state. The ruler is provided for scale, where each tick mark represents 1mm.