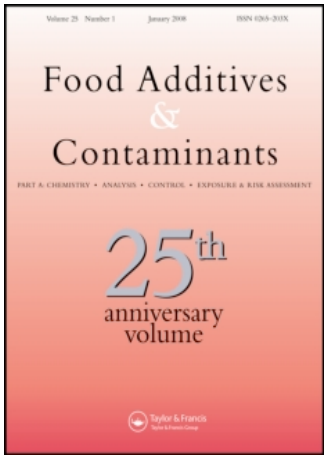

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Reducing human exposure to aflatoxin through the use of clay: A review

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Abstract
Innovative sorption strategies for the detoxification of aflatoxins have been developed. NovaSil clay (NS) has been shown to prevent aflatoxicosis in a variety of animals when included in their diet. Results have shown that NS clay binds aflatoxins with high affinity and high capacity in the gastrointestinal tract, resulting in a notable reduction in the bioavailability of these toxins without interfering with the utilization of vitamins and other micronutrients. This strategy is being evaluated as a potential remedy for acute aflatoxicosis, and as a sustainable human intervention for aflatoxins via the diet. Phase I and II clinical trials confirmed the apparent safety of NS for further study in humans. A recent study in Ghanaians at high risk for aflatoxicosis has indicated that NS (at a dose level of 0.25%) is effective in decreasing biomarkers of aflatoxin exposure and does not interfere with the levels of serum vitamins A and E, and iron and zinc. In summary, enterosorption strategies/therapies based on NS clay are promising for the management of aflatoxins and as a sustainable public health intervention. The NS clay remedy is novel, inexpensive and easily disseminated. Based on the present research, aflatoxin sequestering clays should be rigorously evaluated *in vitro* and *in vivo*, and should meet the following criteria: (1) favourable thermodynamic characteristics of mycotoxin sorption, (2) tolerable levels of priority metals, dioxins/furans and other hazardous contaminants, (3) safety and efficacy in multiple animal species, (4) safety and efficacy in long-term studies, and (5) negligible interactions with vitamins, iron and zinc and other micronutrients.

Keywords: *NovaSil clay, mycotoxins, aflatoxins, aflatoxin-binding agent, aflatoxin sorbent, aflatoxin-sequestering agent, clinical trial, Ghana*

Introduction

Historical perspective on moulds

Moulds (and their metabolic by-products) can be beneficial, as well as harmful, to humans and animals. Moulds have been used since ancient times in the production of various foods including cheese and salami and in the fermentation of beer and wine (Peraica et al. 1999). The secondary metabolites from these same types of moulds have been used as very effective antibiotics for the treatment of disease and as drugs for other important medicinal purposes. For example, the Chinese used moulds for obstetrical purposes nearly 5000 years ago (Hesseltine 1979). Unlike bacterial toxins, the mycotoxins are not protein in nature, but consist of highly diverse organic structures characterized by a variety of heteroatom-containing functional groups. Many of these potent organic chemicals, though invisible to the naked eye, can be found in mould-contaminated food sources and may be harmful if ingested in high enough quantities or over a long enough period of time. Fortunately, the toxicity of these compounds is dose related, and their levels in

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foods and feeds are typically low to non-detectable. However, during extended periods of drought the production of certain hazardous mycotoxins can be unavoidable and may result in contaminated food and feed products that present significant health risks to man and animals (Phillips et al. 2002, 2006; Huebner et al. 2004; Williams et al. 2004).

Mouldy food poisoning and human disease

Although the term 'mycotoxin' was not commonly used until the mid-20th century, earlier records suggest that mycotoxin contamination of food and major outbreaks of disease associated with the consumption of mouldy food have occurred frequently throughout history. A variety of toxic effects of moulds can be traced to very early civilizations, including the Chinese almost 5000 years ago (Ramsbottom 1953; Van Rensburg and Altenkirk 1974). Of the more than 300 mycotoxins that have been identified and chemically characterized, many have been found as contaminants of food and have been linked to the aetiology of disease in humans (and animals). Of these, the aflatoxins have been extensively studied due to their frequent occurrence in foods (especially in developing countries) and their mutagenicity and carcinogenicity (Wogan 1992; Wild and Hall 2000; Wild and Turner 2002).

Aflatoxins

Discovery

There was a lack of understanding of the consequences of aflatoxin exposure on human and animal health, until the early 1960s, when mouldy feed was associated with the loss of thousands of young turkeys in the UK. In this incident, the affected animals showed signs of severe liver necrosis as well as fatty degeneration, fibrosis and extensive bile-duct hyperplasia (Siller and Ostler 1961). Upon investigation it was discovered that the Turkeys had been fed Brazilian peanut meal containing the mould *Aspergillus flavus* along with four metabolic by-products, namely aflatoxins B_1 (AfB_1) (Figure 1), B_2 (AfB_2), G_1 (AfG_1) and G_2 (AfG_2) (Asao et al. 1963). For confirmation the same symptoms were produced in a variety of other species, including ducklings (Sargeant et al. 1961) and rats (Lancaster et al. 1961) following ingestion of the contaminated peanut meal. The dramatic effects of the aflatoxins resulted in significant scientific interest in delineating the chemical structures and toxicological properties of aflatoxins (and other mycotoxins).

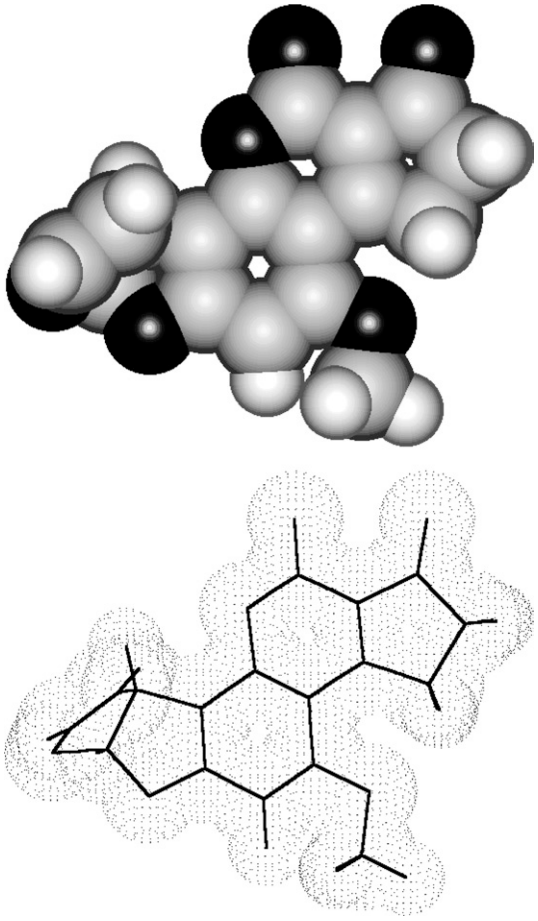

Figure 1. Molecular model of aflatoxin B_1 showing the spatial arrangement of atoms. The molecule is planar, except for the terminal furan which is kinked in the *cis* configuration (coming out of the page). White, hydrogen; dark grey, oxygen; grey, carbon.

Toxic effects

The first observations regarding the toxicity of aflatoxins were recorded during the Turkey X incident in the UK in 1960. In these birds, acute necrosis and bile duct proliferation of the liver was observed (Lancaster et al. 1961). Soon after the same types of symptoms were reported in ducklings and pheasants (Sargeant et al. 1961). A number of acute dosing studies have been performed with AfB_1 to determine LD_{50} values for a wide range of animals (Council for Agricultural Science and Technology (CAST) 1989). While AfB_1 was found to be toxic for many species, some animals were identified as highly sensitive, including duckling, rabbit, and rainbow trout (Muller et al. 1970). Other common symptoms of acute poisoning by AfB_1 include depression and anorexia, as seen in the recent contamination of pet food in South Texas (Garland and Reagor 2001) and South Carolina (Lang 2006). Chronic toxicity studies have also been conducted with lower levels

of aflatoxin exposure. One of the major effects is a general reduction in weight gain for a variety of production animals, including pigs, cattle and poultry. Also, milk production in dairy animals can be decreased in the presence of aflatoxin-contaminated food (CAST 1989) and the milk carries a metabolite of known as AfM_1. This chemical is highly regulated because infants and young children may consume large quantities of milk, and the young of all species are more susceptible (than adults) to the effects of aflatoxins (Leeson et al. 1995).

The main effect of chronic exposure to aflatoxin in humans is hepatocellular carcinoma (HCC). While the incidence of this disease is low in the USA, in parts of Africa and Asia these numbers can be very high (Groopman et al. 1988). Epidemiological studies have shown a positive correlation between the intake of aflatoxin and liver cancer among African and Asian populations. For example, a study by Bulatao-Jayme et al. (1982) showed that the relative risk for liver cancer, when consuming a large level of aflatoxin with a minor amount of alcohol, was 17.5 as compared with a relative risk of 3.9 when alcohol consumption was heavy and aflatoxin intake was light. A diet dependent on foods such as cassava, peanuts, sweet potato and corn increases the likelihood of consuming mould-infested food. A serious confounder in these studies was the high rate of hepatitis B virus (HBV) in these populations. The potential interaction of HBV with aflatoxin is not fully understood (Groopman et al. 1988). The carcinogenic potency of AFB_1 in individuals positive for hepatitis B virus (HBV) surface antigen (HBsAg) has been reported to be considerably higher compared with individuals who are negative for HBsAg. Most of the epidemiological data are from geographical areas where both the prevalence of HbsAg + individuals and aflatoxins are high; the relationship between these risk factors in areas of low aflatoxin contamination and low HBV prevalence is unclear (World Health Organization (WHO) 1998).

Biochemical mode of action

AfB_1 is a direct-acting mutagen and identification of a DNA adduct was made by Essigmann et al. (1977). It was shown that the 8,9 vinyl ether group is transformed to an epoxide through cytochrome P450-mediated oxidation; carbon 8 on aflatoxin reacts with guanine at the N^7 position to form an 8,9-dihydro-8-(N^7-guanyl)-9-hydroxy-AfB_1 adduct (Figure 2). Studies have utilized nuclear magnetic resonance (NMR) to characterize the intercalation and adduct formation of AfB_1 with two oligodeoxynucleotide sequences

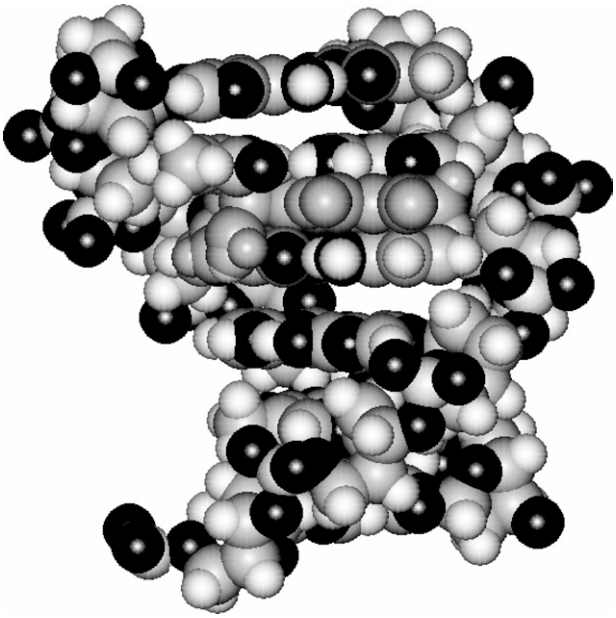

Figure 2. Molecular model showing AfB_1 (in medium grey) intercalated between the strands of DNA.

[d(ATC^{AFB}GAT).d(ATCGAT) and d(AT^{AFB}G-CAT)$_2$] (Gopalakrishnan et al. 1990). Importantly, AfB_1 has been shown to disrupt several genes involved in the growth of cancer. When administered to rats, it was demonstrated that the resulting liver tumours contained an activated form of a proto-oncogene of the c-Ki-ras family. In addition, the hepatocellular tumours of AfB_1-treated rats showed high expression of the proto-oncogenes c-H-ras and c-myc (McMahon et al. 1986, 1987). Proto-oncogenes stimulate growth in the normal cell; when mutated, loss of function leads to rapid growth characteristic of cancer. Tumour suppressor genes provide a complement to this system, keeping growth in check in the normal cell. AfB_1 has been shown to produce a G $\rightarrow$ T transversion in the p53 tumour suppressor gene in human hepatocytes (Aguilar et al. 1993). In addition to DNA and RNA damage, AfB_1 has also been shown to interact with RNA and intercellular proteins. Interactions with protein (Sabbioni et al. 1987; Guengerich et al. 2002a, 2002b) may explain some of the non-carcinogenic effects following exposure to aflatoxin (Eaton et al. 1994).

Consequences of exposure

AfB_1 has been described as a human carcinogen (Group 1A) and implicated in HCC (International Agency for Research on Cancer (IARC) 1976, 1987, 1993). Also, studies suggest that AFs impair the cellular and humoral immune system in animals, and that low-level exposure to these toxins can cause immunosuppression and increased susceptibility to

disease (Rodricks and Stoloff 1977; Miller et al. 1978; Richard et al. 1978; Peska and Bondy 1994; Hinton et al. 2003). A study by Turner et al. (2003) in Gambian children showed evidence that secretory IgA in saliva may be reduced from dietary aflatoxin exposure. This was the first report of immunosuppression in humans associated with AF biomarker measures. Jiang et al. (2005) recently confirmed this finding in adult humans in Ghana and showed a significant correlation between aflatoxin exposure and suppression of the immune system. Other consequences of dietary exposure to aflatoxins include adverse effects on growth and antinutritional effects in animals. For example, AFB_1 has been shown to reduce hepatic vitamin A significantly in a variety of animals, including chickens (CAST 1989; Pimpukdee et al. 2004; Williams et al. 2004). Importantly, Gong et al. (2002, 2004) and Turner et al. (2007) reported an association between biomarkers of aflatoxin exposure and growth impairment in children in West Africa.

Although many countries have regulatory limits for aflatoxins in foods/feeds, outbreaks of poisoning frequently occur. A recent outbreak of aflatoxin poisoning in Kenya resulted in a 39% case fatality rate and was linked to consumption of foods containing toxin levels as high as $8000\,ng\,g^{-1}$ (Centers for Disease Control and Prevention (CDC) 2004). Drought stress exacerbates fungal infection, thus enhancing production of the aflatoxins. This is especially true between a latitude of 40°N and 40°S of the equator, a hot zone which encompasses many developing countries where aflatoxins in the diet of humans and animals are largely uncontrolled (Williams et al. 2004). The poorest people who are most likely to consume foods contaminated with aflatoxins suffer the most severe effects, including disease and even death following acute exposure (Lewis et al. 2005). Additionally, it is estimated that 80% of all HCC cases occur in developing countries (Wild and Hall 2000). Thus, feasible interventions and therapies to diminish human and animal exposure to aflatoxins are imperative; dietary calcium montmorillonite clay, used as an aflatoxin enterosorbent, may provide a practical, cost-effective, and sustainable solution to the problem.

Intervention approaches

Chemopreventive agents

Because avoiding consumption of aflatoxin-contaminated foods for many is simply not feasible, effective means for reducing dietary exposure to aflatoxins are highly desirable (Phillips et al. 2006). Chemoprevention is one strategy used to solve the problem in high-risk populations. This involves the use of natural or synthetic agents to block, retard, reverse or modulate the carcinogenic process (Gupta and DuBois 2001; Sporn and Suh 2002). A variety of chemopreventative agents exist as natural constituents in the human diet; many of these include phytochemicals derived from various sources. Although efficacious against a wide range of carcinogens, most of these compounds occur at very low levels in a nutritionally balanced diet and are poorly absorbed in the gastrointestinal tract (Hayatsu et al. 1988; Dragsted et al. 1993). The chemopreventative agent oltipraz, an antischistosomal drug, has been evaluated for use in humans exposed to dietary aflatoxins in China (Kensler et al. 1999; Wang et al. 1999). In clinical trials oltipraz, when administered to individuals exposed to dietary aflatoxins, increased the level of glutathione *S*-transferase-mediated conjugation of aflatoxin 8,9-epoxide, but also inhibited cytochrome P450 1A2 activity, a key enzyme that activates aflatoxin to the reactive epoxide (Wang et al. 1996, 1999; Kensler et al. 1999). Oltipraz may also inhibit hepatitis B virus (HBV) transcription through elevation of p53 providing an additional contribution to HCC chemoprevention (Chi et al. 1998). Chlorophyllins are natural occurring constituents of the human diet that have been shown to be effective anticarcinogens in several animal models (Dashwood et al. 1998). They are hypothesized to act as interceptor molecules by binding with carcinogens, such as AfB_1, thereby diminishing bioavailability by impeding their absorption. (Breinholt et al. 1995). In a 4-month clinical trial in China, consumption of 100 mg of chlorophyllin at each meal led to an overall 55% reduction in median urinary levels of aflatoxin-N^7-guanine adducts versus the placebo (Egner et al. 2001). Application of these compounds in humans would require careful evaluation including long-term effects of enzyme modulation and potential interferences with the uptake of essential nutrients from the diet. Green tea-derived polyphenols, which are highly effective agents against cancer in various animal models, are also being considered as possible interventions for populations at high risk for HCC. Research has indicated that green tea inhibits the initiation of AfB_1-induced hepatocarcinogenesis in the rat by modulating metabolism of AfB_1 (Qin et al. 1997). Administration of green tea (3% in water) prevented hepatic focal lesion growth induced by dieldrin in B6C3F1 mice (Klaunig and Kamendulis 1999). In humans, inverse associations between the level of green-tea consumption and the risk of development and/or time of cancer onset have also been observed (Nakachi et al. 2000; Fujiki et al. 2002).

Interventions that reduce the dose of aflatoxins from contaminated foods

Food surveillance. Surveillance and subsequent regulation of susceptible commodities, such as groundnuts and maize for aflatoxins and other mycotoxins, are routinely used as a primary intervention to safeguard the health of consumers as well as the economic interests of producers and traders in various countries. These surveillance data are frequently used to establish regulatory guidelines that define the limits of aflatoxins and other mycotoxins in foods. However, in many developing countries, these guidelines are not adequately enforced and result in populations at high risk for aflatoxicosis, i.e. recent outbreak of acute aflatoxin poisoning in Kenya (CDC 2004; Lewis et al. 2005).

Community education. One of the most practical and fundamental interventions at the subsistence-farm level in developing countries, is the use of low-technology approaches, such as community education on food handling and storage, as described by Turner et al. (2005). These primary approaches have been shown to reduce significantly the level of aflatoxin contamination in post-harvest foods and associated exposure in human populations at high risk for aflatoxicosis.

Aflatoxin enterosorption (*NovaSil clay*). Another strategy for reducing food-borne exposure to mycotoxins is the inclusion of various binding agents or sorbents in the diet. Many of these binding agents are purported to prevent the deleterious effects of diverse mycotoxins in a variety of animals (primarily poultry and swine). As early as 1979, adsorbent clay minerals were reported to bind AfB_1 in liquids (Masimango et al. 1979). Additionally, bleaching clays used to process canola oil were found to lessen the effects of T-2 toxin (Carson and Smith 1983; Smith 1984). The dietary consumption of earth (i.e. geophagy) has been observed for centuries and across all continents in both humans and animals (Carretero 2002). Clay eating has been recorded from traditional human societies and is considered 'culturally acceptable' in many African countries and China (Johns and Duquette 1991; Diamond 1999). A practical approach of current interest for the prevention of aflatoxicoses is the incorporation of non-nutritive clay minerals in contaminated food/feed to sorb aflatoxins in the stomach and intestinal tract, thus reducing toxin bioavailability and distribution to the blood, liver and other target organs (Phillips et al. 1995, 2002, 2006; Phillips 1999). Using multiple animal models, the present authors' laboratory has shown that NovaSil^{TM} (NS) clay, a calcium montmorillonite, can prevent the adverse effects of exposure to dietary aflatoxins.

Initially, NS (which was referred to as HSCAS in the early literature), was sold as an anticaking additive for animal feeds. It was reported to sorb aflatoxin B_1 with high affinity and high capacity in aqueous solutions and was shown to rescue broiler and Leghorn chicks from the toxic effects of 7500 ppb aflatoxin in the diet (Phillips et al. 1987, 1988, 2006). In subsequent studies, NS and other similar montmorillonite and smectite clays have been reported to protect against aflatoxin toxicity in a variety of young animals including rodents, chicks, turkey poults, ducklings, lambs, pigs, mink and trout (Phillips 1999; Phillips et al. 1990, 1991, 1994, 1995; Colvin et al. 1989; Bonna et al. 1991; Harvey et al. 1991a, 1991b; 1993, 1994; Voss et al. 1993; Kubena et al. 1990a, 1990b, 1991, 1993; Ledoux et al. 1999; Smith et al. 1994; Marquez and Hernandez 1995; Cerdchai et al. 1990; Lindemann et al. 1993; Abdel-Wahhab et al. 1998; Nahm 1995; Jayaprakash et al. 1992; Ellis et al. 2000). In studies using radiolabelled aflatoxins, NS clay has also been shown to decrease the bioavailability of aflatoxins and reduce aflatoxin residues in poultry (Davidson et al. 1987; Jayaprakash et al. 1992), rats (Sarr et al. 1995; Mayura et al. 1998) and pigs (Beaver et al. 1990). Aflatoxin M_1 levels in milk from lactating dairy cattle and goats were also decreased when NS was included in the diet (Ellis et al. 1990; Harvey et al. 1991b; Smith et al. 1994).

Mechanisms of aflatoxin sorption to NS. The suggested mechanism of AfB_1 sorption by NS is an electron donor acceptor (EDA) mechanism. The platelets of NS clay are negatively charged due to isomorphic substitution, and thus they attract positively charged ions to balance this charge. Compounds with areas of electron deficiencies (partial positive areas) can also be attracted to the platelets (Haderlein et al. 1996). The carbons comprising the dicarbonyl system in aflatoxins are partially positive and have been shown to be essential to the adsorption process. AfB_1 is planar with the exception of the terminal furan (Figure 1). The importance of the spatial orientation of AfB_1 was demonstrated when stereochemical differences of some aflatoxin analogues resulted in significant effects on the tightness of binding. These results also suggested that the sorption of aflatoxin onto NS may favour an orientation where the furan is aligned away from the surface. Adsorption isotherms on heat-collapsed NS have demonstrated that the interlamellar region of NS is the primary site of binding with external surfaces accounting for only minor sorptions of aflatoxins. Based on the

thermodynamics, AfB_1 binds strongly to NS, exhibited by an estimated heat of sorption (enthalpy) of $-50\,KJ\,mol^{-1}$. Interference from compounds with stereochemical restrictive groups could also play an important role in the adsorption process. For the analogues that contain functional groups that make them larger than AfB_1, their insertion, docking and adsorption at surfaces in the interlamellar channel might be restricted. Our results also indicate a good correlation between the magnitude of partial positive charges on carbons C_{11} and C_1 of the β-dicarbonyl system and the strength of adsorption of planar ligands, suggesting an EDA mechanism with the surface of the clay. Other mechanisms of AfB_1 sorption to NS surfaces involve the potential chelation of interlayer cations (especially Ca^{2+}) and various edge-site metals (Grant 1998; Phillips 1999; Phillips et al. 2002, 2006).

Selectivity of NS clay. Research has demonstrated that NS clay has a notable preference (and capacity) for aflatoxins. NS at a level of 0.5% w/w in the diet of poultry did not impair phytate or inorganic phosphorous utilization (Chung and Baker 1990). In other studies in poultry, the addition of NS at concentrations of 0.5% (which is recommended for anticaking in feeds), did not impair the utilization of riboflavin, vitamin A, manganese, or zinc (Chung et al. 1990). NS (0.5% w/w) was also shown to protect young chickens from aflatoxin levels as high as 7500 ppb; these levels are not likely to be found in human food; although, the recent exposure of humans in Kenya was linked to toxin levels as high as 8000 ppb. While clay-based interventions are clearly effective for aflatoxins, the same effectiveness has not been demonstrated for other mycotoxins. Importantly, unmodified NS clays have not been shown to strongly bind other structurally diverse mycotoxins, e.g. zearalenone, deoxynivalenol, T-2 toxin, ochratoxin A, cyclopiazonic acid, ergotamine, and fumonisins, nor do they significantly prevent the adverse effects of these mycotoxins when included in the diet of animals. For example, in enterosorbent studies in poultry with mycotoxins other than aflatoxin, the inclusion of NS clay in the diet did not prevent the adverse effects of cyclopiazonic acid (Dwyer et al. 1997), T-2 toxin (Kubena et al. 1990a), diacetoxyscirpenol (Kubena et al. 1993), ochratoxin A (Huff et al. 1992), and fumonisins (Lemke 2000). The inclusion of clay in the zearalenone-contaminated diets of mink alleviated some fetotoxicity, but did not reduce the hyperestrogenic effects (Bursian et al. 1992). Also the average daily weight gain was unchanged in pigs exposed to deoxynivalenol when clay was added to the diet at 0.5 and 1.0% w/w. The only effective method for decreasing deoxynivalenol toxicity was dilution of the contaminated maize (Patterson and Young 1993). Although *in vitro* tests showed potential for protection of ergotamine toxicity with NS (Chestnut et al. 1992; Huebner et al. 1999), NS at levels of 2.0% w/w did not protect rats or sheep from fescue toxicosis. NS's selectivity was further demonstrated in our laboratory in studies involving nanostructured thin films of NS on quartz that were used as affinity probes for aflatoxins in contaminated media. Our findings show that this composite exhibited comparable selectivity to the Aflatest affinity column from Vicam (Huebner and Phillips 2003; Huebner et al. 2004).

Long-term safety study in rats. In earlier studies in animals, no observable adverse effects from NS were reported following ingestion of doses up to 2.0% w/w in the diet. For example, Sprague–Dawley rats that ingested NS clay at dietary concentrations as high as 2% throughout pregnancy, did not show significant trace metal bioavailability in a variety of tissues and showed neither maternal nor foetal toxicity (Wiles et al. 2004). Since most of our preliminary work was based on short-term exposures not greater than 6 weeks in duration, a long-term exposure was warranted to establish the safety of NS further. Before an adverse events/dosimetry trial in humans, a rodent model was used to evaluate the relative safety of chronic exposure to NS clay in the diet. Male and female Sprague–Dawley rats were fed rations containing 0, 0.25, 0.5, 1.0, and 2.0% levels of NS clay *ad libitum* over a period of 6.5 months. No morbidity or mortality was observed in the animals throughout the study duration. The results of this study indicated that rats treated with 0.25–2% NS clay in the diet did not exhibit dose-dependent or NS-related adverse effects on body weight gains, feed conversion ratios, relative organ weights, gross anatomy and histological appearance of major organs, haematology, and serum biochemistry parameters. Additionally, levels of selected nutrients including vitamins A and E, Fe, and Zn were unaffected (Afriyie-Gyawu et al. 2005). Given the safety and efficacy of NS, as demonstrated in a variety of animal models, it was hypothesized that NS-based interventions might be beneficial for the treatment of humans who are at high risk for aflatoxicosis (Phillips et al. 2006).

Adverse events/dosimetry trial with NS in humans. As a precursor to a phase IIa clinical intervention trial with NS in Ghana, a short-term (2-week) safety evaluation of NS was carried out at Texas Tech University in 50 healthy adults (Wang et al. 2005). The overall design followed the guidelines

for a randomized and double-blind phase I clinical trial. This study was conducted: (1) to evaluate the short-term safety and tolerance of NS capsules in normal human subjects; and (2) to establish optimal protocols for human intervention studies. NS capsules were produced under sterile conditions using US Good Manufacturing Practices. Also, the NS was sterilized at 121°C before encapsulation. Preceding the chronic animal and short-term human studies, NS was analysed for concentrations of various environmental contaminants, including priority toxic metals and dioxins/furans to ensure compliance with federal and international standards. A total of 50 adults, ages 20–45, who met the recruiting criteria were randomly divided into two study groups. The high-dose group (HD) took three capsules of NS three times a day (a total of 3.0 g), and the low dose group (LD) took three capsules of NS three times a day (a total of 1.5 g). All capsules were of the same colour and size. The two dose levels were extrapolated from previously published dosimetry data from animal studies (Phillips 1999; Phillips et al. 2002, 2004; Afriyie-Gyawu et al. 2005). After 14 days of capsule ingestion, NS (up to 3.0 g day^{-1}) was considered safe for further human studies based on physical examination, biochemistry and haematology results. Concentrations of the standard parameters analysed after the trial were statistically similar to those levels determined before trial. Also, no significant difference was observed for any reported adverse symptoms between LD and HD groups. This study confirms the selectivity of NS clay for aflatoxins, in that no statistical differences were observed in the levels of serum vitamins A and E, and iron and zinc in the participants after 2 weeks of NS ingestion. This evidence further confirms that NS demonstrates binding specificity for aflatoxins and lack of interaction with vitamins A and E. The adverse events trial provided the basis for the phase IIa human intervention study at the Ejura-Sekyedumase district (ESD) of the Ashanti region of Ghana, West Africa.

Screening of clays in Ghana for aflatoxin binders. Before the initiation of a 3-month clinical intervention trial in Ghana, 73 'edible' clays from the marketplace at ESD and 11 clays used in the ceramic industry from other locations in Ghana were tested for aflatoxin sorption using isothermal analyses in our laboratory. Our rationale for screening clays near the study site in Ghana was to identify those that were similar to NS clay for subsequent human studies. It was hypothesized that study participants could be categorized as geophagic or non-geophagic in the context of aflatoxin exposure. However, upon analysis of 84 samples from different geographic locations in the country, we found that none of these sorbed aflatoxins with high affinity and capacity (Figure 3).

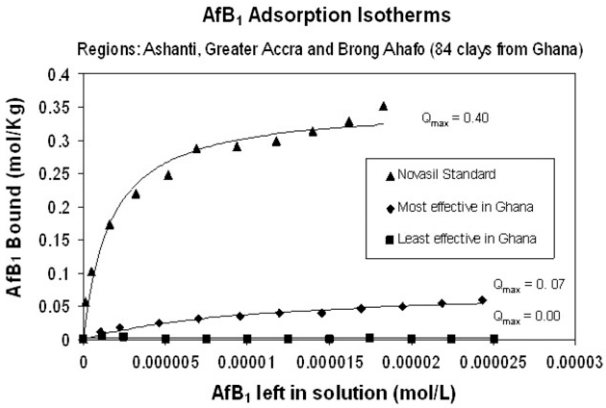

Figure 3. Representative isothermal plots of the most effective and least effective clay samples from Ghana for the sorption of AfB_1. These plots are compared with a standard isotherm for NovaSil. The most effective sample from Ghana was obtained from the Brong/Ahafo Region ($Q_{max} = 0.07$), and the least effective sample was obtained from the Ashanti Region ($Q_{max} = 0.00$). None was comparable with NovaSil ($Q_{max} = 0.40$), and would not be expected to decrease the bioavailability of AfB_1.

Phase IIa clinical intervention trial with NS in Ghana. Aflatoxin contamination in food products remains a serious burden in the developing world where a lack of untainted food supplies and poverty present a major and persistent challenge to many people in affected areas (McAlpin et al. 2002; Shephard 2003). Avoiding consumption of aflatoxin-contaminated foods is one of the most fundamental approaches for reducing risk of aflatoxicosis in humans. However, this is simply not feasible for many communities in developing countries and therefore emphasizes the need for viable intervention strategies to manage aflatoxin contaminated diets and treat aflatoxicosis. A recent study involved a 3-month double-blind and placebo controlled, phase IIa clinical trial conducted in the Ejura-Sekyedumase district, Ashanti Region, Ghana (Afriyie-Gyawu et al. 2007). The objective was to evaluate the safety, efficacy, and tolerance of dietary NS when administered to humans for the prevention of aflatoxin exposure and toxicity. The study protocol was approved by the Institutional Review Boards of Texas A&M University and its counterpart in Ghana for Ethical Clearance. Five hundred and seven volunteers were clinically screened to evaluate their general health, pregnancy status, and blood AfB_1-albumin adduct levels, and 177 of them were enrolled as study participants. Subjects were randomly assigned to three groups: high-dose (HD),

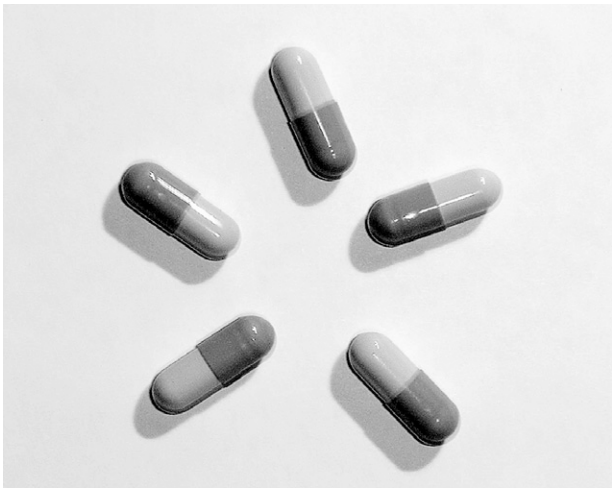

Figure 4. NS capsules for the phase IIa clinical intervention trial in Ghana. Treatment doses were 0, 1.5 or 3.0 g NS day^{-1} before meals with water (microcrystalline cellulose was used as a placebo).

low-dose (LD) and placebo-control (PL) groups that received 3.0, 1.5 and 0 g NS day^{-1}, respectively, in capsules (Figure 4). To ensure compliance to treatment regimens and participant well-being, trained study monitors supervised administration of the encapsulated NS to participants and recorded side effects daily. On-site physicians performed physical examinations monthly. Blood and urine samples were collected for laboratory analysis. Over 90% of the participants completed the study, and compliance rate was more than 97%. Also, 99% of the time participants reported no side effects throughout the study. Mild to moderate adverse health events (approximately 0.5% of the time) were recorded in some participants but none of them appeared to be associated with NS treatment. No NS-related, significant differences were shown in haematology, liver and kidney functions, and electrolytes among the three groups. In the serum biochemical analysis, isolated statistical differences in a few parameters were detected but no trends of association or dose-dependency were observed, and were all within the normal physiological ranges (Afriyie-Gyawu et al. 2007).

This study represents the first phase IIa clinical intervention trial to evaluate the safety and efficacy of NS clay in human subjects. Results suggest that short-term inclusion of NS in the diet at a minimal effective dose (MED) of 0.25% (w/w) would not likely produce overt toxicity in humans. Moreover, the results of this study support the application of NS for the management of aflatoxicosis in humans who are acutely exposed to high levels of dietary aflatoxins. Additional results from this study indicate that ingestion of capsules containing an MED of NS, significantly reduce biomarkers of aflatoxin exposure in the blood and urine from study participants (Wang et al. 2007). Further studies are planned to optimize the dosimetry and delivery methods for NS clay. Also, phase IIb, phase III intervention, and epidemiological studies are needed to confirm the safety and efficacy of NS for long-term therapy and the potential inclusion in foods for humans in areas at high risk for aflatoxicosis.

Summary

NS clay is commonly used as an anti-caking agent in animal feeds. Importantly, its inclusion in feed (at relatively low levels) may also serve to protect animals by tightly sorbing aflatoxins in the stomach and intestines resulting in decreased bioavailability. Based on numerous studies, it is anticipated that NS clay-based enterosorption of aflatoxins in animals will result in improved growth rates, feed conversions and general health along with diminished aflatoxin residues in foods of animal origin (such as milk).

Our recent findings from clinical intervention trials with NS are of particular relevance to populations in developing countries where the incidence of HCC and adverse health impacts from frequent exposures to aflatoxin are often elevated. Moreover, the use of NS for the protection of humans that are at high risk for HCC and aflatoxicosis appears to be culturally acceptable and sustainable. Eventually, the preferred delivery of NS may be through its inclusion in salt (like iodine), taking advantage of its anticaking properties, or as an additive in common groundnut and maize-based foods. Extensive research with NS clay and other sorbent materials suggests that potential mycotoxin enterosorbents (e.g. chemical and biological binders and/or sequestering agents) should be rigorously evaluated *in vitro* and *in vivo*. These should meet the following criteria:

- Favourable thermodynamic characteristics of sorption.
- Tolerable levels of priority metals, dioxins/furans and other hazardous substances.
- Safety and efficacy in multiple animal species.
- Safety and efficacy in long-term studies.
- Negligible interactions with vitamins, iron and zinc.

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