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Abbreviations:

Aflatoxin B₁: AFB₁

Hepatocellular Carcinoma: HCC

Chlorophyll: chl_a

Chlorophyllin: CHL

Accelerator Mass Spectrometry: AMS

Area under the curve: AUC

Dibenzo(a,l)pyrene: DBP

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Abstract (250 words)

Chlorophyll (Chla) and chlorophyllin (CHL) were shown previously to reduce carcinogen bioavailability, biomarker damage, and tumorigenicity in trout and rats. These findings were partially extended to humans (*Proc Natl Acad Sci U S A* **98**, 14601-14606 (2001)), where CHL reduced excretion of aflatoxin B₁ (AFB₁)-DNA repair products in Chinese unavoidably exposed to dietary AFB₁. However, neither AFB₁ pharmacokinetics nor Chla effects were examined. We conducted a small unblinded crossover study to establish AFB₁ pharmacokinetic parameters in human volunteers, and to explore possible effects of CHL or Chla co-treatment on those parameters. For protocol 1, fasted subjects received an IRB-approved dose of ¹⁴C-AFB₁ (30 ng, 5 nCi) by capsule with 100 ml water, followed by normal eating and drinking after hr 2. Blood and cumulative urine samples were collected over 72 hr, and ¹⁴C-AFB₁ equivalents were determined by Accelerator Mass Spectrometry. Protocols 2 and 3 were similar except capsules also contained 150 mg of purified Chla, or CHL, respectively. All protocols were repeated 3 times for each of three volunteers. The study revealed rapid human AFB₁ uptake (plasma k_a $5.05 \pm 1.10 \text{ hr}^{-1}$, T_{max} 1.0 hr) and urinary elimination (95% complete by 24 hr) kinetics. Chla and CHL treatment each significantly impeded AFB₁ absorption and reduced C_{max} and AUC's (plasma and urine) in one or more subjects. These initial results provide AFB₁ pharmacokinetic parameters previously unavailable for humans, and suggest that Chla or CHL co-consumption may limit the bioavailability of ingested aflatoxin in humans, as they do in animal models.

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Introduction

Aflatoxin is a potent, naturally occurring carcinogenic mycotoxin that is associated with the growth of two types of mold: *Aspergillus flavus* and *Aspergillus parasiticus*. Food and food crops most prone to aflatoxin contamination include corn and corn products, cottonseed, peanuts and peanut products, tree nuts, and milk. Humidity, high temperatures, and other environmental conditions such as insect infestation can encourage aflatoxin growth on crops. Consequently, aflatoxins can invade the food supply at anytime during production, processing, transport and storage. Evidence of acute aflatoxicosis in humans has been reported primarily in developing countries lacking the resources to effectively screen aflatoxin contamination from the food supply (1).

Because aflatoxins, particularly aflatoxin B₁ (AFB₁), are potent carcinogens in some animals, there is interest in the effects of long-term exposure to low levels of these important mycotoxins on humans. Epidemiological studies in Asia and Africa revealed a positive association between dietary aflatoxins and liver cancer (2). High levels of aflatoxins in combination with infection with hepatitis B appear to act synergistically to increase risk of hepatocellular carcinoma (HCC) (3). Intervention programs have been directed toward reducing hepatitis virus infection through immunization and screening of foods for aflatoxin levels. There is also interest in addition of dietary agents that may reduce the bioavailability of aflatoxins by binding in the intestine and limiting absorption, or by increasing metabolism and reducing systemic exposure. Intervention agents currently under investigation include apiginin clays, Oltipraz and chlorophyll or its derivative (4-8). Chlorophyll and its derivatives have a long, well-known history of uses for medicinal and therapeutic preparations (9). Both natural chlorophyll (Chla) and its water soluble derivative sodium copper chlorophyllin (CHL) have been extensively studied for a variety of significant biological activities (10-14). A double-blinded, placebo-controlled intervention trial in Qidong, People's Republic of China, demonstrated that CHL intervention can reduce aflatoxin-DNA adduct excretion among individuals in a population at high risk for liver cancer (8). This study clearly demonstrated a decrease in a biomarker of AFB₁-DNA damage in

individuals chronically exposed to relatively high doses of aflatoxin through their diet. The mechanisms that produce this effect in humans are undefined but may include limiting absorption in the intestine, systemic complexation with bioavailable forms of CHL, or induction of phase II enzymes leading to increased aflatoxin metabolism (7, 15, 16). Preclinical studies in rats and rainbow trout suggested that CHL acts primarily by binding certain aflatoxins, heterocyclic amines and polycyclic aromatic hydrocarbons to reduce bioavailability, genomic damage, and tumor induction (17-19).

Microdosing pharmacokinetic studies are increasingly being used in early translational, or Phase 0, human studies to evaluate the kinetics of compounds at subtherapeutic/subtoxic doses. By taking advantage of sensitive methods such as Accelerator Mass Spectrometry (AMS) for quantifying drug concentrations, kinetic parameters can be evaluated in humans, and new approaches to medical intervention can be tested safely (20). AMS brings three distinct advantages to biochemical research: relevant chemical doses, very small sample sizes, and very low radioactive exposures due to long lived isotopes and/or high levels of isotope dilution.

The purpose of the present study was to investigate AFB₁ pharmacokinetics in human volunteers using microdosing techniques and AMS analysis, and to explore possible effects of Chla and CHL co-treatment on AFB₁ pharmacokinetic parameters. The kinetics of low dose AFB₁ were investigated in three volunteers who received a 30 ng dose of ¹⁴C-labeled aflatoxin, without or with CHL or Chla co-treatment. AMS was used to measure the levels of aflatoxin equivalents in plasma and urine following oral administration. Pharmacokinetic modeling of absorption and disposition was performed to evaluate the disposition of AFB₁ at doses that are within the acceptable levels found in foods according to limits set by the USDA (21).

Results

AFB₁ pharmacokinetics: Urine excretion over the 72-hour period for AFB₁ challenge with and without intervention is presented in Figure 1 as total cumulative excretion for each subject. Excretion was rapid with greater than 95% of the total urine AFB₁ equivalents produced within the first 24 hours. As seen in

Figure 2, absorption of AFB₁ equivalents into systemic circulation was also rapid with peak concentrations achieved within approximately 1 hour. Non-compartmental calculations are presented in Table 2 for all subjects with a breakdown of individual pharmacokinetic parameters presented in Table 3. The parameters presented for AFB₁ demonstrated that absorption of AFB₁ equivalents into plasma follow a first-order process. A two-compartment model with first-order input and elimination was fitted to each plasma data set, yielding parameters that are also presented in Tables 2 and 3. These data demonstrated that AFB₁ equivalents fit a two-compartment model of absorption and elimination with a rapid distribution phase followed a slower elimination phase.

Effects of Chla and CHL intervention: Responses to intervention with Chla and CHL were qualitatively similar among the three volunteers, but with some evidence for inter-individual variability in degree of response. AFB₁ equivalents excreted over 72 hour are presented in Figure 1 and reveal a consistent trend toward reduced urinary excretion. The effects were significant for Chla intervention in all volunteers (P<0.01 for volunteer 1 and P<0.05 for volunteers 2 and 5). Although CHL showed a similar trend toward protection, intervention reached significance in volunteer 5 (P<0.01) but not subjects 1 (P = 0.08) or 2 (P = 0.08). Subject 1 excreted an average of 29.0 ± 2.31% of the dose in the first 72 hours when given AFB₁ alone, while intervention reduced the amount of the initial AFB₁ dose to 13.3 ± 5.10% for Chla and 24.0 ± 2.95% for CHL. Similar effects were seen in subject 2 with an average of 34.4 ± 5.60% for AFB₁, 22.4 ± 2.17% for Chla, and 24.1 ± 5.37% for CHL, while subject 5 excreted 33.3 ± 2.79% of the AFB₁ dose, 21.4 ± 4.94% with Chla, and 21.2 ± 2.17% with CHL.

Changes in plasma concentrations of AFB₁ equivalents following intervention in each subject mirrored those seen in urine. Apparent in Figure 2 is a consistent trend for reduction in mean plasma concentrations during the initial absorption phase of the plasma concentration time curve. These data were fit to a two compartment model as described in Methods, and individual pharmacokinetic parameters of aflatoxin equivalents are presented in Table 3. For some of the profiles following AFB₁ alone or with CHL intervention, it was determined that a two-compartment model was over-parameterized; thus, for these cases, a one-compartment model with first-order input and first-order

output was fit to the data. As seen in Table 3, Subject 1 demonstrated a robust and highly significant response to Chla intervention that resulted in significant reductions in C_{max} ($P < 0.01$) and AUC_{0-24} hours ($P < 0.01$). The response to CHL by this subject was less pronounced although changes in T_{max} ($P < 0.01$) and the absorption rate constant ($P < 0.05$) were significant. Subject 2 response was less robust and was not found to be significant for changes in C_{max} ($P = 0.27$) or AUC_{0-24} ($P = 0.33$) although a similar pattern of reduced plasma AFB₁ equivalents was observed and the absorption rate constant was significantly reduced with both Chla ($P < 0.05$) and CHL ($P < 0.05$) intervention. Subject 5 also responded to both Chla and CHL intervention with a significant reduction in C_{max} ($P < 0.05$) and, for Chla, a significant change in AUC_{0-t} and AUC_{0-24} ($P < 0.05$). Figure 2 also includes the plasma data for subject 3. Although this subject did not complete the study, the one intervention trial with CHL gave a result consistent with protection by this agent.

Discussion

AFB₁ pharmacokinetics in human subjects: The intestinal absorption of AFB₁ at levels commonly found in the diet had not been previously measured in humans. The present study utilized AMS techniques, which permitted an investigation of the absorption and pharmacokinetics of AFB₁ and its metabolites in humans, using doses of ¹⁴C and AFB₁ that fall within an allowable range of safety. We did not attempt in this initial study to discriminate between free AFB₁ and its various metabolites and conjugates, which limits interpretation relating to potential inter-individual or treatment-related differences in metabolic capability. Based on total ¹⁴C equivalents, AFB₁ was rapidly absorbed into plasma in all volunteers with first-order kinetics. The k_a of $5.05 \pm 1.10 \text{ hr}^{-1}$ is similar to the *in situ* absorption rate constant in the rat of $5.84 \pm 0.05 \text{ hr}^{-1}$ reported by Ramos and Hernandez (22). Following absorption, AFB₁ equivalents were rapidly cleared following a first-order process in our volunteers with a $t_{1/2\alpha}$ (harmonic mean) of 2.86 and a $t_{1/2\beta}$ (harmonic mean) of 64.4 hours. The $t_{1/2\alpha}$ that we obtained is consistent with excretion patterns reported for AFB₁ in Rhesus monkey by Wong and Hsieh (23) that suggest terminal excretion of AFB₁ is primarily through the kidney, where 40% of the

dose being eliminated as either unbound (~30%) or chloroform extractable from the urine (~10%) within the first 24 hours. Although we could not distinguish between AFB₁ and its metabolites or conjugates in this study, we speculate that the α -phase is likely free aflatoxin while the β -phase is most likely aflatoxin conjugated to albumin and other plasma proteins. The long β -phase observed is not surprising considering that, in the monkey, 100 hours following administration of AFB₁, 13.6% of the dose remained in the liver with 5.8% remaining in the plasma suggesting that a significant fraction of the dose has a long residence time (23). This is also consistent with reports of the linear formation of AFB₁-albumin and AFB₁-DNA adducts over an 80,000-fold dose range when Fischer rats were challenged with AFB₁ (24). The patterns of excretion observed in our study suggest a similar phenomenon occurs in humans as in rats with small amounts of aflatoxin found in the diet distributed throughout the body in roughly the same proportions as would be expected from larger doses.

Effects of CHL and Chla on AFB₁ uptake and distribution: Many studies have characterized the molecular dosimetry of AFB₁ as a measure of possible carcinogenic effects in the absence and presence of intervening agents (23, 25-27). One such agent, CHL, has been characterized extensively for its cancer chemopreventive potential in animals and humans (8, 17, 28-30). More recently, cancer chemopreventive effects of natural chlorophyll itself have been reported in rodent and fish models, through mechanisms that involve reduced systemic carcinogen uptake (17, 18). The present study now extends these findings by establishing the kinetics of low-dose aflatoxin absorption in each of four human volunteers, and the effects of CHL and Chla intervention on specific AFB₁ pharmacokinetic parameters in three of those subjects. The results show that intervention by CHL and Chla produced similar, and frequently significant, effects on AFB₁ uptake and distribution among all individuals. In all individuals, Chla intervention produced a significant 40-60% reduction in excretion of urinary aflatoxin equivalents (Fig. 1). For CHL, the extent of protection in subject 5 was identical to that provided by Chla. A similar but non-significant trend for CHL protection was seen for subject 2, and to a lesser extent for subject 1. We interpret these results as direct evidence for CHL- and Chla-mediated reductions in systemic uptake of aflatoxin in humans, as in preclinical models (19, 27, 28). An

alternative interpretation could be that a single co-treatment with either chlorophyll species alters AFB₁ metabolism in a direction that would reduce urinary excretion, for instance through rapid induction of phase II detoxication pathways (16). This interpretation seems highly unlikely, especially since interference effects become apparent within 30 minutes of co-treatment, but is not formally ruled out by this study.

The plasma data are largely consistent with what was observed in urine, and support the notion that CHL and Chla limit AFB₁ uptake from the human GI tract. Evidence for this hypothesis would be provided by intervention-mediated reductions in k_a , C_{max} , and initial AUC, increases in T_{max} , and absence of effect on elimination rate. As seen in Table 2, CHL and Chla intervention produced mean pK values consistent with this set of expectations for 33 of the 36 possible intervention-control comparisons. These apparent changes are, however, statistically significant in only 8 of the 36 possible comparisons with controls (AFB₁-only). Interpretation of the AUC data is especially complicated by the fact that our AMS detection approach did not distinguish among the free and bound states of parent AFB₁ and its metabolites. Thus the plasma evidence for reduction in systemic AFB₁ uptake by co-treatment with chlorophylls is consistent with, but less compelling than, that for urine data in this pilot study. Future design expansions might include more sampling from 0-1 hr, to more precisely determine k_a and T_{max} values, and use of separation methods to distinguish among the bound and unbound aflatoxins, which would improve sensitivity to detect intervention effects.

Alternative mechanisms of protection by chlorophylls: A number of recent reports suggest that certain phytochemicals, as well as the chlorophyll derivative chlorophyllin, may act by triggering induction of metabolic processes that reduce AFB₁ toxicity and genomic damage. However, studies with animal models for AFB₁ carcinogenesis in our laboratory have provided evidence inconsistent with such a mechanism for CHL or Chla chemoprotection in the whole animal. For example, extensive dietary pretreatment of rainbow trout with a high (and otherwise chemopreventive) dosage of CHL, followed by a single aqueous treatment with AFB₁, failed to reduce AFB₁-DNA adduction or hepatocarcinogenesis, under conditions where CHL co-treatment with AFB₁ was effective (31). This

result showed that pre-loading the animal with CHL, even at a dose sufficient to turn the liver, skin, and bile green, did not provide any form of induction that could protect against AFB₁. Instead, CHL and AFB₁ co-exposure was necessary and sufficient for protection. In a second study with this model, CHL co-treatment was shown to significantly reduce the bioavailability of AFB₂, an AFB₁ analogue that interacts equally as strongly with CHL *in vitro* but lacks the 8,9 double bond essential to be genotoxic or carcinogenic (19). In the case of this simply defined metabolic profile, CHL co-treatment produced significantly reduced AUC and C_{max}, and delayed T_{max}, which can be interpreted as definitive evidence supporting the reduce bioavailability hypothesis *in vivo*. This result demonstrates that metabolic changes are not required to observe reduced uptake and altered pharmacokinetics of aflatoxins. A more recent study in the rat (17) revealed that co-treatment with either CHL or Chla significantly elevated fecal excretion of AFB₁ equivalents, reduced hepatic AFB₁-DNA adduction and reduced preneoplastic lesions in the liver and colon in the absence of any detectable induction of hepatic phase II enzyme activities *in vivo*. In two companion studies, dietary CHL and Chla provided strong protection against dibenzo(a,l)pyrene (DBP) uptake and tumorigenesis in the rainbow trout, without significant alterations in gene expression by microarray analysis ((32); unpublished observations). Indeed, the only observed molecular effect of these chlorophylls was to reverse the dose-responsive alterations in expression that were induced by the carcinogen itself, as one would expect if the primary mechanism *in vivo* were simple reduction in carcinogen bioavailability. In sum, these studies provide convincing evidence that chlorophylls can strongly inhibit uptake of aflatoxins and polycyclic aromatic hydrocarbons in the whole animal, in the absence of any detectable alterations in phase II activities.

Finally, we note that treatments were not completely randomized in the present study design. We elected to first administer three cycles of AFB₁ alone (protocol 1) to each subject, which was then followed by six treatment cycles that were randomized to include three each of protocols 2 and 3. This procedure assured that prior treatments with CHL or Chla could not have modified the baseline results with AFB₁ alone. Under protocols 2 and 3, CHL or Chla were given along with AFB₁, not before, again to eliminate the possibility that prior treatment might provoke enzyme induction. Figure 2 demonstrates

that protective effects against AFB₁ uptake were evident well within 30 minutes of CHL or Chla treatment, a time-scale that does not favor a requirement for alterations in metabolic enzyme expression. This time-scale is potentially compatible with inhibition of hepatic enzyme catalysis as a protective mechanism, but data are lacking to establish if rapid inhibition of metabolic enzymes could take place under the conditions of this study, and if so, would produce the effect of reduced appearance of AFB₁ equivalents in urine and plasma. Based on present data, we conclude that the changes in pharmacokinetic parameters due to Chla and CHL co-treatment reflect an ability of these blocking agents to reduce the amount of aflatoxin absorbed in the intestine, in humans as well as animal models.

While our results are consistent with reduced uptake as the primary protective mechanism, the precise mechanism through which this occurs remains not established. A number of previous studies have indicated that Chla as well as CHL can form strong non-covalent complexes *in vitro* with AFB₁, DBP, heterocyclic amines, and other carcinogens with at least some partial planarity and aromaticity. This interaction, and formation of a macromolecular complex with carcinogen, may be expected to reduce the rate of carcinogen uptake from the intestine, as has been demonstrated directly for CHL *in vitro* using the Caco2 monolayer transport model (29) and *in vivo* using the rat intestinal loop absorption model (32). We have not, however, envisioned a means to detect or quantify such complexes in the intragastric milieu *in vivo*, and so cannot be certain of their mandatory participation in chemoprevention. We also note some indication of inter-individual variability in response to CHL, with subject 1 appearing less responsive to protection than the other subjects. This would not be expected if reduced uptake were solely the result of intragastric CHL-AFB₁ interaction. Potential explanations include variable gastric environments that might alter the strength of such interactions, or polymorphisms in certain proteins that might provide variability in the ability of CHL to mediate uptake (e.g. transport proteins) or alter phase I metabolism (e.g. CYP3A4). In addition, the rarity of plasma 0-72 hr AUC reduction by CHL (and Chla) seems at odds and not consistent with our former study (19) of AFB₂ pharmacokinetics in the trout. However, interpretation is limited by lack of information on the identity of equivalents,

including parent compound and its bound and unbound metabolites. It is noteworthy that the previous China CHL study (7) lacked any demonstration that sustained CHL intervention altered steady-state plasma aflatoxins. Additional studies will be essential to more fully define and elucidate mechanisms of protection by chlorophylls in human subjects.

Materials and Methods

Chemicals. [^{14}C]AFB₁ (Moraveck Biochemicals, Inc.) was a generous gift of Ronald T. Riley (R.B. Russell Research Center, Athens, GA). Radiochemical and chemical purity was confirmed to be >97% by HPLC equipped with an online-radioisotope detector and a photodiode array detector. The specific activity was determined to be 50mCi/mmol by LSC. Chla was isolated from spinach and purified by counter current chromatography following our published method (33). CHL (Rystan, Little Falls, NJ), was the same lot used in the China intervention trial (7). The lactose and gelatin capsules were obtained from the College of Pharmacy at Oregon State University (OSU).

Capsule preparation. Capsules were prepared by filling the cap with lactose and adding 50 μL [^{14}C]AFB₁ in ethanol (0.6 ng/ μL , 0.1 nCi/ μL). For the intervention, 150 mg of Chla in ethanol or 150 mg of powered CHL were added to the cup of the capsules. For [^{14}C]AFB₁ and chlorophyll, the ethanol was allowed to evaporate before the capsules were sealed. Each capsule was prepared fresh as needed.

Human volunteers. *Design:* The protocol design for this study, and all revisions, were approved by both Institutional Review Boards (IRB) at OSU and Lawrence Livermore National Laboratory (LLNL). Participants were recruited from a group with sufficient scientific knowledge to evaluate the toxicological issues and risks of the study. Written informed consent was obtained from all enrolled volunteers. After the consent form was read and signed and any questions were answered, the volunteers provided their medical history and underwent a limited physical examination. Volunteer characteristics are provided in Table 1. Permission for the administration of [^{14}C] radiolabeled aflatoxin was obtained from the OSU Radiation Safety Committee. Information was also obtained on any medication that the subjects received which may have affected the metabolism and disposition of [^{14}C]AFB₁. All clinical procedures

were conducted with the technical assistance of the Integrated Environmental Health Sciences Clinical core within the Environmental Health Sciences Center at OSU. *Compliance:* Subjects 1, 2, and 5 completed the entire study in full compliance with the specified protocols. Subject 3 did not complete the study and subject 4 was enrolled into the study but excluded before the first dose due to illness. The first AFB₁-only exposure trial conducted with subject 1 was a range-finding experiment that resulted in a slight revision for all subsequent experiments and subjects. Specifically, three additional urine collections and blood draws were added and the study period was increased from 36 to 72 hours to allow for a more complete pharmacokinetic analysis.

Study design. The initial design called for serial treatment of three volunteers. Three protocols were used: *Protocol 1* - At 8 AM fasted subjects were administered a gelatin capsule containing ¹⁴C-AFB₁ (30 ng, 5 nCi) swallowed with 100 ml water. Normal eating and drinking was resumed at 10 AM, beginning with a specified and provided breakfast. Blood (3 ml) was drawn by a qualified nurse at times 0, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 3, 4, 8, 12, 24, 48 and 72 hours. Each volunteer was given the choice between a catheter (time points 0 to 12 hours) and multiple venipunctures. Total urine samples were collected at intervals of 0 – 2 hr, 2 – 4 hr, 4 – 8 hr, 8 – 12 hr, 12 – 24 hr and every subsequent 24 hr until the end of study. This protocol was repeated three times to obtain baseline data for each subject. *Protocol 2* - Nominally, a similar protocol was followed, except that the capsule also contained 150 mg of purified Chla . This protocol was repeated three times. *Protocol 3* - Protocol 1 was followed, except that the capsule also contained 150 mg of CHL. This protocol was also repeated three times. The order of protocol treatments was not totally randomized in this pilot study. Each subject received the three exposure cycles for Protocol 1 (AFB₁ alone) in succession, in order to establish baseline AFB₁ pharmacokinetic data in the absence of possible carryover effects from CHL or Chla intervention. Initial data indicated that ¹⁴C doses were adequate for detection, and that clearance rates in plasma and urine were sufficiently rapid that exposure intervals of seven days or greater could be conducted without interference from baseline carry-over. Volunteers then received the three CHL and three Chla interventions, which were given in differing orders within and among subjects to minimize the potential

that one intervention might bias the subsequent treatment. Each subject thus received nine test treatments: AFB₁ alone (repeated three times), with Chla (repeated three times), and with CHL (repeated three times). Subjects were free-living (i.e., not confined to the testing facility) during each 72-hour study period.

AMS analysis. Plasma and urine samples were shipped to LLNL where AMS was used to measure the ratio of ¹⁴C to total carbon. Specifically, samples were thawed at room temperature, and an aliquot of each sample (typically 20 μL blood and 100 μL urine) were placed in quartz tubes and dried in a vacuum centrifuge. The carbon content of each sample was converted to CO₂ by combustion, and the CO₂ was quantitatively reduced to graphite in the presence of zinc and titanium hydride, condensing onto cobalt at approximately 500 °C for 4 hours (34, 35). ¹⁴C/C ratios (units of amol ¹⁴C/mg C) in the graphite samples were then quantified using the 1 MV accelerator mass spectrometer at LLNL. The ¹⁴C/C ratios in the samples were converted to ¹⁴C contents (amol ¹⁴C/mL urine or plasma) using the density and carbon contents of the plasma and urine samples (36). For plasma samples, the value used for carbon content were assumed to be 4.2 % (37). As the percentage carbon is more variable for urine, each individual urine sample was analyzed at LLNL for carbon content, using an Exeter 440 CHN Analyzer. Densities of the plasma and urine samples were assumed to be 1g/cc. For each human subject dosing ¹⁴C carbon contents of “time 0” plasma and urine samples were subtracted from the ¹⁴C carbon contents of the other samples. Excess ¹⁴C concentration was converted to aflatoxin equivalents (parent compound and all metabolites) using the specific activity of the dose (50 mCi/mmol), together with the compound molecular weight of 312.

Pharmacokinetic Analysis. Pharmacokinetic parameters of AFB₁ equivalents were first calculated using noncompartmental methods. Urine excretion over time, maximum plasma concentration (C_{max}), and time to C_{max} (T_{max}) were determined by observation from the collected concentration verses time data. WinNonlin (v. 4.1.a) was used to perform noncompartmental analysis for estimation of kinetic parameters for aflatoxin with and without intervention. Area under the curve (AUC) was calculated for the intervals [0-t], [0-8], [0-24], and [0-inf], where t = time of the last measurable concentration and inf =

infinity, using the linear trapezoidal method. Differences between control and intervention groups were analyzed using one-way ANOVA followed by Dunnett's multiple comparison test and were considered significant for P values less than 0.05. Analysis of urine data beyond the assessment of cumulative excretion for AFB₁ with and without intervention was not performed because of the inability to distinguish between free AFB₁ and its metabolites. Additionally, in order to estimate absorption and disposition rate constants, a two-compartment model with first-order input and first-order elimination was fit to each of the plasma concentration-time profiles using WinNonlin (v. 4.1.a) software. Estimated parameters included A and B (coefficients, or zero-time intercepts), k_a (absorption rate constant), alpha and beta (disposition rate constants), and T_{lag} (absorption lag time). For plasma data, because the order could not be completely randomized, linear in order trends were examined as possible alternate explanations for observed treatment effects. In only one analysis (C_{max} within subject 5) was there such evidence due to both an overall strong linear trend coupled with similar trends within the replicate runs for each treatment group.

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Figure legends:

Figure 1. Cumulative urinary excretion of aflatoxin equivalents in subjects 1, 2, and 5. Aflatoxin amount is derived from AMS analysis based on total ¹⁴C. Total aflatoxin excretion at 72 hours was assessed by one-way ANOVA followed by Dunnett's multiple comparison test comparing AFB₁ alone to interventions for each subject. Chla intervention reduced AFB₁ excretion in subject 1 (P<.01) and subject 5 (P<.05). CHL intervention significantly reduced AFB₁ excretion in subject 5 only.

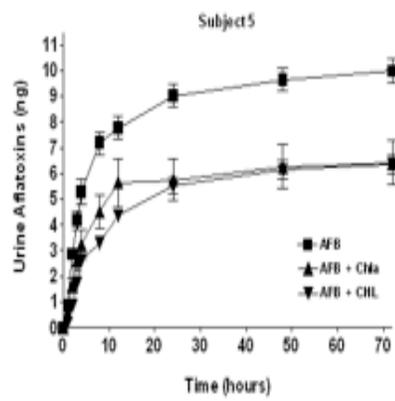
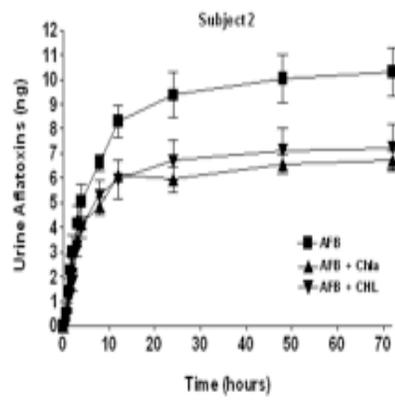
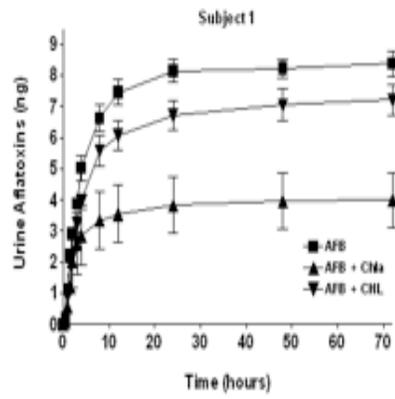
Figure 2. Pharmacokinetic profiles of aflatoxin equivalents from plasma samples collected at 0.25, 0.5, 0.45, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 8.0 hr. Aflatoxin concentration is derived from AMS analysis based on total ¹⁴C from aliquots of plasma samples.

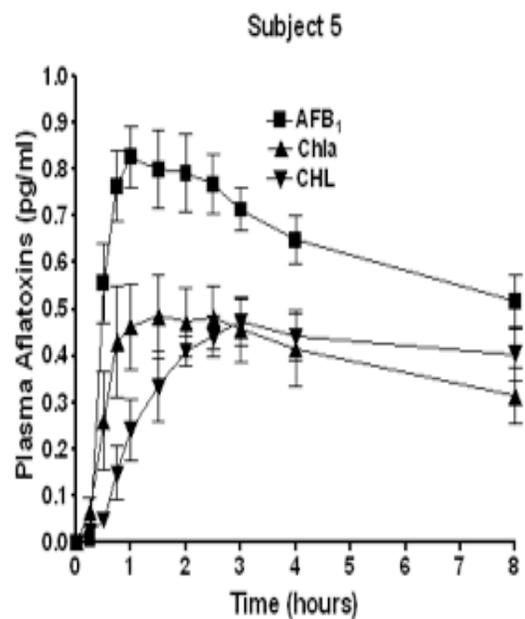
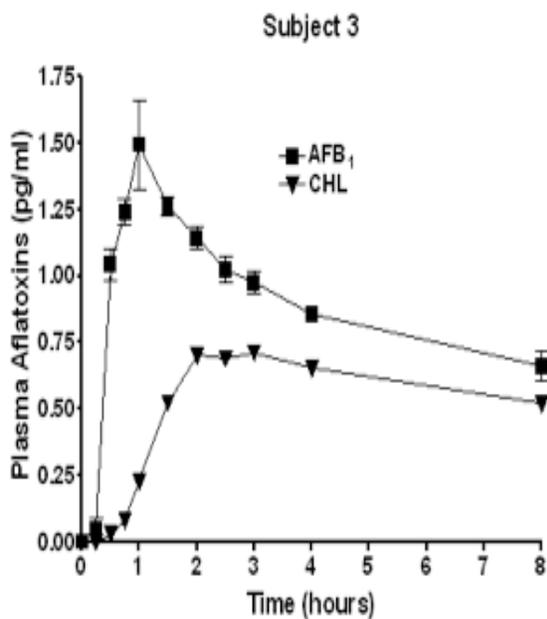
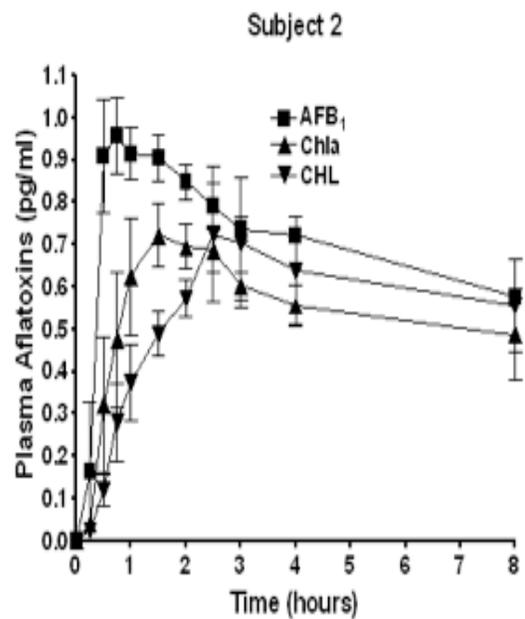
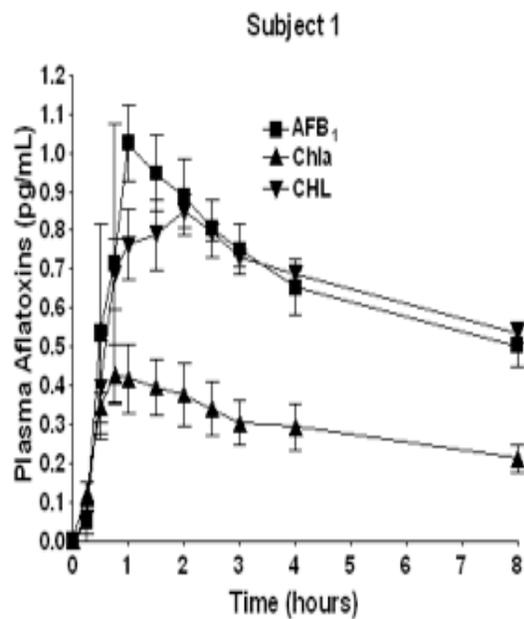
Table legend:

Table 1: Study subject information.

Table 2: Mean pharmacokinetic parameters of AFB₁ equivalents in plasma.

Table 3: Individual pharmacokinetic parameters of aflatoxin equivalents. Mean and SD over (N=) 3 replicate runs except where noted in parentheses.





Subject	Gender	Height	Weight	Age
1	Male	6'2"	180	65
2	Male	6'0"	197	46
3	Female	5'4"	138	32
5	Male	6'4"	212	59

Aflatoxin			
Parameter	Unit	Mean \pm SD	CV%
C_{max}	pg/mL	0.941 \pm 0.154	16.3
T_{max}	Hr	1.02 \pm 0.31	30.7
AUC_{0-t}	hr*pg/mL	25.6 \pm 6.3	24.7
AUC_{0-inf}	hr*pg/mL	54.7 \pm 18.7	34.3
AUC_Extrap	%	50.9 \pm 8	15.7
AUC_{0-8}	hr*pg/mL	5.4 \pm 0.7	13.2
AUC_{0-24}	hr*pg/mL	12.4 \pm 1.8	14.7
CL/F	L/hr	0.66 \pm 0.39	59.9
V_d/F	L	62.3 \pm 7.8	12.5
k_a	hr ⁻¹	5.05 \pm 1.10	19.5
λ	hr ⁻¹	0.24 \pm 0.06	24.4
β	hr ⁻¹	0.01 \pm 0.003	26.8
$t_{1/2}$ (harmonic mean)	Hr	2.86	--
$t_{1/2}$ (harmonic mean)	Hr	64.4	--

		Aflatoxin Alone	With Chlorophyll	With Chlorophyllin
Subject 1	C _{max} (pg/mL)	1.02 ± 0.17	**0.44 ± 0.14	0.86 ± 0.09
	T _{max} (hr)	0.99 ± 0.02	0.83 ± 0.15	**1.86 ± 0.33
	t _{1/2λ} (hr)	2.06 ± 0.28	3.15 ± 1.50	2.93 ± 1.46
	k _a (absorption rate constant)	4.78 ± 0.01 (2)	3.97 ± 0.45 (2)	*2.54 ± 0.60
	AUC _{0,t} (hr*pg/mL)	22.57 ± 9.93	10.27 ± 3.40	27.20 ± 2.69
	AUC _{0,24}	11.94 ± 2.51	**4.97 ± 1.61	12.55 ± 1.25
	Subject 2	C _{max} (pg/mL)	0.97 ± 0.15	0.73 ± 0.13
T _{max} (hr)		0.92 ± 0.51	1.67 ± 0.77	4.16 ± 3.34
t _{1/2λ} (hr)		4.39 ± 2.83	8.26 (1)	7.75 ± 2.66 (2)
k _a (absorption rate constant)		7.76 ± 1.13 (2)	*3.28 (1)	**0.97 ± 0.47
AUC _{0,t} (hr*pg/mL)		29.00 ± 1.05	21.87 ± 3.71	23.83 ± 8.30
AUC _{0,24}		13.40 ± 0.77	9.60 ± 3.02	11.15 ± 3.51
Subject 5		C _{max} (pg/mL)	0.83 ± 0.12	*0.50 ± 0.13
	T _{max} (hr)	1.16 ± 0.29	2.35 ± 0.79	2.17 ± 0.77
	t _{1/2λ} (hr)	3.74 ± 0.26	3.67 ± 1.14	2.96 (1)
	k _a (absorption rate constant)	3.43 ± 0.40	2.91 ± 1.43	1.27 ± 1.08
	AUC _{0,t} (hr*pg/mL)	25.23 ± 5.34	*14.10 ± 4.77	17.77 ± 3.80
	AUC _{0,24}	11.88 ± 2.07	*6.42 ± 1.56	8.17 ± 1.47

(1) and (2) denotes N=1 and N=2 respectively, because either two or one of the replicate runs could not be fit to a two compartment model.

* denotes p<.05 and ** denotes p<.01 versus Aflatoxin alone (Dunnett adjusted).