Dietary Aflatoxin Exposure and Chemoprevention of Cancer: A Clinical Review

Daniel L. Sudakin, M.D., M.P.H. *

Department of Environmental and Molecular Toxicology, Oregon State University, Corvallis, Oregon, USA

ABSTRACT

Exposure to dietary aflatoxins is considered to be an important risk factor for the development of hepatocellular carcinoma in certain regions of the world. Significant advances have recently been made in understanding the clinical toxicology of aflatoxins. These include the development and validation of biomarkers of exposure and genotoxic effect. These biomarkers are currently being utilized to explore the potential that pharmaceutical interventions may have in modifying the toxicokinetics of dietary aflatoxin exposure. Preliminary results of clinical trials with the drug oltipraz suggest that it may modify the genotoxic effects of aflatoxin B1 by inhibiting bioactivation pathways and stimulating detoxification pathways. More recent results of a clinical trial with chlorophyllin suggest that this drug may have a role in preventing dietary exposure to aflatoxin B1 by reducing its oral bioavailability. The preliminary results of these chemoprevention studies may ultimately have implications for cancer prevention in high-risk populations in the future.

Key Words: Aflatoxin; Mycotoxin; Toxicokinetics; Chemoprevention; Oltipraz; Chlorophyllin.

INTRODUCTION

Food represents an unavoidable source of human exposure to certain mycotoxins. Both acute and chronic health effects have been described from food-borne pathways of exposure to high levels of aflatoxin. As information has accumulated on the clinical toxicology of aflatoxins, preventive strategies aimed at reducing human health risks have been implemented. These include good agricultural practices, good management practices, and regulations specific to aflatoxins in foods and animal feed. More recently, pharmaceutical strategies have been investigated to evaluate the potential that they may ultimately reduce human health risks in...
Aflatoxins represent a family of structurally related difuranocoumarin derivatives produced primarily by certain species of *Aspergillus flavus* and *Aspergillus parasiticus*. These toxigenic fungal species are distributed throughout the world, but are more prevalent in warm, sub-tropical and tropical climates in comparison with temperate environments (1). The production of aflatoxins by fungi is complicated and difficult to predict, being affected by environmental factors (temperature, humidity, growth substrate) and strain-specific genetic factors. The chemical form of aflatoxin that has been determined to be of greatest potency and human health significance is aflatoxin B₁ (AFB₁, Fig. 1). Acute dietary exposure to AFB₁ has been implicated in epidemics of acute hepatic injury (2,3), and in certain regions of the world with high levels of dietary exposure AFB₁ has been associated with a high incidence of primary hepatocellular carcinoma (HCC) (4). The International Agency for Research on Cancer has concluded that there is sufficient evidence for the carcinogenicity of AFB₁ in humans (Group I) (5). A synergistic interaction between dietary exposure to AFB₁ and chronic infection with Hepatitis B has been reported in some epidemiological studies of HCC (6–8).

**AFLATOXINS**

The oral bioavailability of dietary aflatoxins has not been systematically measured in human studies. The metabolic pathway for absorbed AFB₁ appears in Fig. 2. The liver is the primary site of biotransformation of ingested aflatoxins. The predominant human CYP450 isoforms that are involved in human metabolism of AFB₁ include CYP3A4 and CYP1A2. Both enzymes catalyze an epoxidation reaction to AFB₁, which forms a highly reactive intermediate, AFB₁ exo-8,9-epoxide (9). CYP1A2 is also capable of catalyzing a hydroxylation reaction of AFB₁ to form aflatoxin M₁ (AFM₁), which is a poor substrate for epoxidation (9), less potent than AFB₁ (10), and generally considered a detoxification metabolite.

The formation of AFB₁ exo-8,9-epoxide is a critical step in the genotoxic pathway. This molecule is highly unstable and binds with high affinity to guanine bases in DNA to form aflatoxin-N⁷-guanine (11). It is also capable of binding to lysine residues in serum albumin (AFB-albumin) (12–15), as well as other cellular proteins (16). In addition to conversion to AFM₁, detoxification pathways for AFB₁ involve conjugation reactions of AFB₁ exo-8,9-epoxide with glutathione (GSH) resulting in the formation of AFB-mercapturic acid (AFB-NAC), in a reaction catalyzed by glutathione S-transferase (16).

Animal studies and in vitro human studies suggest that AFB₁ has a short elimination half-life of less than 60 minutes (17). Longer elimination half-lives of approximately 8 hours have been reported for aflatoxin-N⁷-guanine adducts (18), AFM₁, and AFB-NAC conjugates (19). The half-life of serum AFB₁-albumin is approximately 2 to 3 months, which is consistent with the half-life of circulating albumin (14). The relative contribution of urinary and biliary excretion has not been systematically studied in humans, although one study of children with high levels of dietary exposure found AFB₁ and other aflatoxin metabolites more frequently and in higher quantities in feces than in urine (20). The role of enterohepatic recirculation is not known.

The genotoxic effects resulting from metabolic activation of AFB₁ have been studied at the molecular level. Aflatoxin-N⁷-guanine can give rise to guanine (purine) to thymine (pyrimidine) transversion mutations in DNA (21). In vitro studies of animal and human tissues as well as epidemiological studies of HCC have reported a high incidence of this specific transversion mutation occurring at codon 249 of the p53 tumor suppressor gene (22–25), a region corresponding to

**Figure 1.** Aflatoxin B₁ (AFB₁).
the DNA binding domain of the corresponding protein. A recent meta-analysis reported a positive association between the prevalence of p53 tumor suppressor gene transversion mutations at codon 249, and increasing levels of dietary aflatoxin exposure among individuals with primary HCC (26).

BIOMARKERS OF DIETARY EXPOSURE TO AFB1

A combination of analytical techniques including immunoaffinity chromatography and HPLC have been utilized in human biomarker studies investigating the relationship between aflatoxins and HCC (19,27). Indicators of recent exposure to aflatoxin include urinary aflatoxin-N7-guanine and AFM1, both of which show a dose-dependent relationship between dietary intake and excretion of AFB1 (12,19,28). A single 24-urine specimen may not be adequate to measure recent exposure to aflatoxin, because significant variation in dietary intake and urinary excretion over the course of several days has been documented (28). More recent studies of human urine samples have detected and quantified AFB-NAC, the product of Phase II metabolism of AFB1 (29).

Because the longer half-life of circulating albumin, serum AFB-albumin adducts have also been evaluated as a biomarker of chronic exposure to aflatoxin in epidemiological studies (14). A positive correlation between dietary aflatoxin intake and AFB-albumin adducts in the serum has been demonstrated (12,15). In a recent epidemiological study, a positive association was reported between measurements of AFB-albumin adducts in the serum and cases of HCC among individuals infected with Hepatitis B (30).

As dietary exposure to aflatoxins is considered to be a significant risk factor in the high incidence of HCC in certain regions of the world, considerable effort has been made toward identifying interventions to reduce these
risks. These have included pharmaceutical or dietary interventions aimed toward blocking or modifying the susceptibility of humans to the actions of carcinogens, a strategy that has been defined as chemoprevention or chemoprotection (31). The remainder of this review will focus on chemoprevention studies that are currently being conducted in humans, which are focused on dietary aflatoxin exposure and HCC.

OLTIPRAZ AND CHEMOPREVENTION

Over the past 15 years, oltipraz (a substituted 1,2-dithiole-3-thione, Fig. 3) is a drug that has been the subject of many investigations in cancer chemoprevention. It is structurally similar to the dithiolethiones found in cruciferous vegetables, which may have a role in cancer prevention (32). Oltipraz was originally developed as a treatment for schistosomiasis and extensively evaluated in human studies (33). Although effective, interest in this drug declined when less expensive alternatives with fewer adverse effects were developed. In the process of studying the pharmacokinetics of oltipraz in animal models, this compound was found to be a potent inducer of enzymes maintaining reduced glutathione stores, including glutathione transferases (34). Pretreatment with dithiolethiones of mice exposed to acetaminophen and carbon tetrachloride was found to reduce or prevent hepatic glutathione depletion and liver damage, and these effects were associated with increased activities of glutathione-S-transferase (35). In a randomized chemoprevention study in which low levels of dietary AFB1 exposure were administered prior to and in combination with oltipraz or placebo, the group receiving dietary oltipraz was found to have complete protection against hyperplastic nodules and hepatocellular cancer (36). Subsequent experimental animal studies of oltipraz administered after chronic dietary AFB1 exposure found similar protective effects against the development of hepatic aflatoxin-DNA adducts, effects which were associated with a significant increase in glutathione-S-transferase activity (37).

The mechanistic pathway of chemoprevention in these studies was hypothesized to occur as a result of reducing the formation of aflatoxin-N7-guanine adducts by stimulating the pathway of detoxification of the reactive AFB1 exo-8,9-epoxide toward glutathione conjugation and away from DNA.

Further research on oltipraz in humans has been conducted since that time. In healthy volunteer studies, the bioavailability of oltipraz has been found to be significantly affected by dietary factors. Under fasting conditions, negligible plasma concentrations of oltipraz have been measured after oral dosing (38). In contrast, increased bioavailability and a lower time to peak serum concentration has been measured when oltipraz was orally administered with a high-fat meal (24%) in comparison to a low-fat meal (less than 5%) (38). Elimination half-lives ranging from 5.6–6.8 hours have been measured after single doses of 125 and 500 mg, respectively (39). At least nine metabolites of oltipraz have been characterized, and their elimination occurs chiefly through urinary excretion (32). At least one of these metabolites, oltipraz M2, retains the biological activity of the parent compound (40).

Pharmacodynamic effects of oltipraz have been investigated in single- and chronic-dose studies in humans. In one study of patients at high risk of colon cancer, subjects receiving single oral doses of 125- and 250 mg/m² were found to have an increase in mean glutathione-S-transferase activity in colon tissue (41). These effects were not present in individuals receiving higher doses (500–1000 mg/m²), but all patients receiving oltipraz were found to have increased gene expression of detoxification enzymes (including gamma-glutamylcysteine synthetase, the initial enzyme in the GSH synthetic pathway) in lymphocyte and colonic tissues (41).

In another study of chronic daily low dose of oltipraz (20–125 mg per day for six months) among individuals at high risk of colon cancer, a significant association between plasma oltipraz concentrations and lymphocyte glutathione levels was measured, although there were no significant correlations between plasma oltipraz concentration and lymphocyte glutathione-S-transferase levels, or percent change in colonic tissue glutathione or GST (42). The study was not randomized and did not include a control arm. Adverse effects included gastrointestinal symptoms (nausea, bloating, flatus), phototoxicity, thermal sensitivity, and paresthesias. These findings are consistent with previous reports of adverse effects associated with oltipraz (32).

More recent study of the pharmacodynamic effects of oltipraz in humans has led to additional mechanistic

![Figure 3. Oltipraz chemical structure.](image-url)
Dietary Aflatoxin Exposure and Chemoprevention of Cancer

considerations of its potential role in chemoprevention. Animal studies and in vitro studies of human liver tissues have reported that oltipraz has inhibitory activity on certain Phase I enzymes, including CYP1A2 and CYP3A4 (43,44). In a healthy human volunteer study, the oral administration of oltipraz (125 mg) for 8 consecutive days was associated with a significant reduction in CYP1A2 activity (45). Inhibition of CYP1A2 activity persisted for 48 hours from the time of last dose, and fully recovered within 14 days in study subjects.

The studies of oltipraz in humans have suggested a potential preventive role in aflatoxin-induced carcinogenesis through different mechanisms (Fig. 2). By inhibiting the activity of CYP1A2 and CYP3A4, metabolic activation of AFB1 to AFB1-8,9-epoxide may be prevented, which could decrease the formation of aflatoxin-N7-guanine adducts. The inductive effects of oltipraz on glutathione-S-transferase activity could reduce the potential for genotoxic injury to hepatic DNA by stimulating the detoxification of AFB1-8,9-epoxide through conjugation with glutathione.

OLTIPRAZ AND HUMAN CHEMOPREVENTION STUDIES

Mechanistic studies of oltipraz and its potential to modify the toxicokinetics of AFB1 have led to the design of clinical trials to evaluate its safety and efficacy in high-risk populations. One such trial (the Oltipraz Chemoprevention Trial) has been conducted in Qidong, Jiangsu Province, in the People’s Republic of China (46). This is a region with a high incidence of hepatocellular carcinoma, as well as chronic dietary exposure to high levels of AFB1.

The Oltipraz Chemoprevention Trial was a randomized, placebo-controlled, double-blind investigation designed to determine the safety and efficacy of oltipraz in reducing biomarkers of aflatoxin exposure (46). Individuals who were pregnant, had chronic disease, hematologic or abnormal blood chemistry values were not eligible for inclusion. The study population included individuals with serological evidence of Hepatitis B surface antigen who had evidence of normal liver function. All study participants had detectable aflatoxin–albumin adducts in the serum. A total of 234 individuals were randomized to receive 125 mg of oltipraz daily, 500 mg of oltipraz weekly, or placebo. The duration of the intervention was eight weeks. Biomarkers of exposure to aflatoxins were collected biweekly during the intervention period, and at eight weeks postintervention. The endpoints evaluated were AFM1 and AFB-NAC excretion in the urine, and serum AFB-albumin adducts.

Of 234 randomized participants, 132 (56%) were compliant with the dosing schedule for the full intervention period (46). Lower compliance rates were observed among the treatment groups receiving 125 mg (47%) and 500 mg oltipraz (50%). Compliance with the protocol for biomarker measurements was good, with 77% and 78% of subjects submitting urine and serum samples according to study protocol, respectively. The incidence of adverse effects was significantly higher among treatment groups receiving oltipraz, with the majority of reports occurring early in the intervention period. Adverse effects were responsible for more withdrawals from the study than other reasons. A syndrome of numbness, tingling, and occasional extremity pain was described among 11.5% of study participants, with a significant increase in reporting frequency in the oltipraz treatment groups in comparison to placebo. In all cases, symptoms resolved within 1 week of discontinuation of the study drug. Elevation of liver function tests were noted among 49 study participants (only one of whom was HBsAg positive), with a similar incidence across all treatment arms. In 37% of these participants, liver function tests had not returned to baseline levels by the end of the study period.

The results of biomarker analyses for the treatment groups are summarized in Table 1. After four weeks of the intervention, the administration of 125 mg oltipraz daily did not result in a statistically significant difference in AFM1 urinary excretion (29). The group receiving 500 mg oltipraz weekly had a statistically significant 51% reduction in the median urinary AFM1 level in comparison with the other treatment groups. No significant difference in AFB-NAC excretion was noted among individuals receiving 500 mg oltipraz weekly, but a significant elevation in the median urinary AFB-NAC.

<table>
<thead>
<tr>
<th>Aflatoxin biomarker</th>
<th>Placebo</th>
<th>125 mg daily</th>
<th>500 mg weekly</th>
</tr>
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<tbody>
<tr>
<td>Afm1 (urine)</td>
<td>No effect</td>
<td>No effect</td>
<td>Decrease</td>
</tr>
<tr>
<td>AFB-NAC (urine)</td>
<td>No effect</td>
<td>Increase</td>
<td>No effect</td>
</tr>
<tr>
<td>AFB-albumin (serum)</td>
<td>No effect</td>
<td>No effect</td>
<td>Triphasic response</td>
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Table 1. Biomarker results of oltipraz chemoprevention study.
excretion was observed among individuals receiving lower doses of 125 mg oltipraz daily.

Biomarkers of chronic exposure to aflatoxin, measured as serum AFB-albumin adducts, revealed no significant differences within the treatment group receiving 125 mg oltipraz daily. In contrast, a triphasic response was observed among individuals receiving 500 mg oltipraz weekly. No effect was observed during the first month of the intervention, but a significant reduction in AFB-albumin adducts was measured between the 5th and 13th weeks of the intervention. A partial rebound of AFB-albumin adduct levels was observed in the second month after the intervention period. When all of the treatment groups were compared, no significant differences in biomarker trajectories of AFB-albumin adducts were observed.

Several explanations for the biomarker findings have been proposed in this study. An inductive effect on Phase II glutathione-S-transferase detoxification has been suggested to explain the elevation in urinary AFB-NAC excretion in the treatment group receiving 125 mg oltipraz daily (29). In contrast, the observation of a significant reduction in urinary excretion of AFM1, and lack of increased excretion of AFB-NAC in the group receiving a higher dose of 500 mg oltipraz weekly have been hypothesized to occur as a result of a masking effect caused by inhibition of CYP1A2 in the formation of the reactive AFB1 epoxide. The inhibitory effects on CYP1A2 in the activation of AFB1 have also been suggested as an explanatory factor in the downward trends in AFB-albumin adducts observed in the treatment group receiving 500 mg oltipraz weekly (47).

While the results of this well-designed intervention appear promising, there remain some uncertainties regarding the interpretation of the results. If induction of glutathione-S-transferase were explanatory for some of the study findings relating to enhanced AFB-NAC excretion, variations among study subjects of the genotypes for glutathione-S-transferase would be anticipated to be an effect modifier (47). Analyses of genotypes for glutathione-S-transferase (class M1 and T1) were performed on the subjects, and no significant relationship was found between null and positive genotypes and either baseline aflatoxin biomarker values or biomarker findings in any of the treatment groups. Other factors that could present difficulties in the interpretation of urinary and serum biomarkers of aflatoxin exposure have been described, including the potential for short-term variations in the dietary intake of AFB1 to influence the results, and variation in the precision of the analytical methodology, as well as the timing of biomarker analyses (longitudinal vs. cross-sectional assessment of samples) (19,48).

The Oltipraz Chemoprevention Trial provides evidence to support the mechanistic studies suggesting that pharmaceutical interventions can modify the toxicokinetics of exposure to dietary AFB1. It is unclear at this time whether these effects can be sustained, and whether they translate into a reduced risk of HCC. In addition to these unanswered questions, future studies on oltipraz and chemoprevention will have to consider the plausibility of implementing such a pharmaceutical intervention on a population-wide basis. The risk of adverse effects and altered pharmacokinetics of other common drugs metabolized by CYP1A2 and CYP3A4 would need to be carefully considered. As studies on oltipraz continue, additional chemoprevention strategies that are mechanistically based, inexpensive, and could be implemented across wide sections of high-risk populations are currently being investigated.

CHLOROPHYLLIN

Sodium copper chlorophyllin (CHL, Fig. 4) is a bright green, water-soluble chemical that is derived from natural chlorophylls which are found in green plants. Chlorophyllin is derived by extraction from crude chlorophyll through a reaction with methanolic sodium hydroxide, followed by replacement of the central magnesium atom with a copper atom. The resulting mixture contains several different chlorin compounds, including predominantly copper chlorin e6 and copper chlorin e5 (49). Chlorophyllin has been available in the United States as an over-the-counter drug (Derifil) for

Figure 4. Sodium copper chlorophyllin.
controlling urinary, fecal, and body odor in geriatric and ostomy patients. It is also FDA-approved as a color additive for use in certain drugs and cosmetic products.

The antimutagenic activities of CHL have been studied in short-term genotoxicity assays both in vitro and in vivo, and the results of these studies have demonstrated potent protection against several classes of mutagens, including aflatoxins, polycyclic aromatic hydrocarbons, and heterocyclic amines (50). Early mechanistic studies had consistently demonstrated that CHL and other chlorophylls form reversible complexes with planar aromatic compounds in vitro (51,52), and these interactions were hypothesized to have chemopreventive effects by limiting the bioavailability of these compounds from the gut. Other in vitro and in vivo studies of animal and human tissues have reported antioxidant effects and nonspecific inhibition of cytochrome P450 activity (53,54).

A more recent investigation of dietary CHL and aflatoxins in a rainbow trout model has reported evidence to support the mechanistic hypothesis of chemoprevention occurring from decreased bioavailability of aflatoxins from dietary CHL (55), in contrast to the target organ effects that have been hypothesized for oltipraz (modulation of Phase I and Phase II enzyme activities). In this study, inclusion of CHL in a water bath containing AFB1 resulted in decreased AFB1 bioavailability, and concurrent dietary exposure to CHL was associated with reduced hepatic AFB1-DNA adduct formation and incidence of HCC. No significant effects on hepatic Phase I or Phase II enzymes were apparent after high-dose dietary exposure to CHL in the rainbow trout model.

CHLOROPHYLLIN AND HUMAN CHEMOPREVENTION STUDIES

The results of the in vitro and in vivo studies on the anti-mutagenic and anti-carcinogenic studies on CHL, in combination with the history of its safe over-the-counter use in humans, have led to interest in exploring its potential chemopreventive effects in populations at high risk of HCC from chronic dietary aflatoxin exposure. The results of a randomized, double-blinded chemoprevention trial of CHL in a sample of residents of Qidong, People’s Republic of China, have recently been published (The Chlorophyllin Clinical Trial) (18). Similar to the Oltipraz Chemoprevention Study, the study population included healthy adults with detectable serum aflatoxin–albumin adducts at baseline. Subjects were randomized to receive either 100 mg CHL three times per day, or placebo. The intervention occurred over a 16-week period, during which 12-hour urine samples were collected every 4 weeks from the study subjects. Serum samples were also collected at baseline and periodically through the intervention period, for the evaluation of routine blood chemistries and alanine aminotransferase activity. In contrast to the Oltipraz Chemoprevention Study, which evaluated AFM1 and AFB-NAC in urine and AFB-albumin adducts in serum, the primary endpoints that were measured in this investigation were urinary aflatoxin-N7-guanine adducts, biomarkers of genotoxic effect.

Excellent compliance with the study protocol was reported, with over 90% of participants adhering to the treatment regimen and biomarker collections (18). The randomized groups were similar in terms of demographics, body mass, Hepatitis B serology, and baseline measurements of aflatoxin–albumin adducts. Biomarker analyses of urine samples at week 12 of the intervention revealed a statistically significant decrease in the median level of aflatoxin-N7-guanine in the group receiving 100 mg CHL daily. In 38% of urine samples collected at week 12, aflatoxin-N7-guanine was not detectable, with no significant difference in nondetects between treatment groups. When the analysis of biomarker data collected at week 12 were restricted to those samples with detectable values, a 49% reduction in the geometric mean levels of aflatoxin-N7-guanine in the urine was measured, results that were statistically significant.

No adverse effects were reported among study subjects. Some participants reported dark-green colored feces. Interestingly, when the investigators regrouped the serum samples serially by subject identification number at the completion of the trial, they noticed that the serum became increasingly green in color. Subsequent analyses were undertaken to determine the source of the tint, and it was found to be a previously unreported copper chlorin e4 ethyl ester (CuCle4 ethyl ester) in the chlorophyllin formulation and copper chlorin e4 (CuCle4) (56). The bioavailability of known and previously unreported chlorins in a commercially prepared CHL formulation was an unexpected finding, given the relative instability of natural chlorophylls to factors such as acid and heat (57). A more recent investigation simulating digestion conditions in human intestinal cells has confirmed the stability of CuCle4 and its efficient uptake by enterocytes, confirming the plausibility of its gastrointestinal absorption and distribution to peripheral tissues (58). These observations raise the possibility that in addition to the role of CHL in reducing the bioavailability of dietary AFB1, additional mechanistic
considerations resulting from systemic absorption of chlorins in CHL may still need to be considered. These observations also emphasize the importance of using well-defined, pure compositions of CHL to avoid difficulties interpreting results from commercially prepared CHL, where batch-to-batch variability has been reported to be considerable (59,60).

SUMMARY

In contrast to other mycotoxins for which the human health data from ingestion pathways of exposure are relatively incomplete, recent studies of AFB1 have provided a significant amount of information that can be utilized to evaluate exposure and response at the individual as well as the molecular level. These advances have led to the investigation of preventive strategies that may influence the toxicology of AFB1 from dietary pathways of exposure. The clinical trials with oltipraz and CHL provide preliminary evidence that pharmaceutical interventions may favorably alter the toxicokinetics of AFB1 through different mechanistic approaches. The extent to which these interventions may ultimately modify the risk of hepatocellular cancer in high-risk populations is an area in need of further research. Through the longitudinal and concurrent assessment of biomarkers of exposure, genotoxic effect, and individual susceptibility to dietary AFB1, future studies will provide clarity to our understanding of the safety and efficacy of these chemopreventive agents.

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