Natural chlorophyll inhibits aflatoxin B1-induced multi-organ carcinogenesis in the rat

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Introduction

Chronic exposure to aflatoxin B1 (AFB1) is a major cause of the high prevalence of hepatocellular carcinoma (HCC) in Asia and sub-Saharan Africa (1,2). Prevention measures that limit human exposure to AFB1, and its adverse health effects, are important public health goals in these geographies. Research in experimental chemoprevention has been a productive means to address these goals. For instance, experiments in animal models such as the rat have demonstrated more than a score of natural and synthetic chemopreventive agents that can dramatically inhibit the formation of aflatoxin biomarkers and the resulting incidence of putative pre-neoplastic lesions and HCCs (3–9).

One such agent, chlorophyllin (CHL), a water soluble, sodium–copper salt of chlorophyll (Chl) (Figure 1), was tested in a clinical chemoprevention trial on a human population in eastern China with chronic, unavoidably high aflatoxin exposure and a high incidence of HCCs (10). Administration of 100 mg dietary CHL three times per day led to a highly significant, 50% reduction in the level of aflatoxin–N7-guanine (AFB–N7-guanine) in the urine of participants (11). Elevated urinary output of this hepatic DNA adduct biomarker in humans is clearly associated with increased risk of liver cancer (12), and diminished levels of AFB–N7-guanine have been associated with reduced HCC risk in several animal studies (3–5,8). Thus, diet supplementation with CHL might reduce human liver cancer risk from dietary AFB1.

Protection by CHL, however, is not limited to AFB1. Mechanistic studies in mammals and fish suggest that CHL acts as a carcinogen-blocking agent, at least in part, by forming tight complexes with AFB1, as well as heterocyclic amine and polycyclic aromatic hydrocarbon carcinogens (13–15). Complex formation appears to block carcinogen absorption, thereby reducing bioavailability to the target tissue resulting in less DNA adduction and, ultimately, lower tumor incidence (16). Additional mechanisms of CHL protection have been proposed including inhibition of bioactivating enzymes, induction of detoxifying enzymes and direct antioxidant activity (16).

While CHL is widely manufactured, purity among different suppliers varies greatly (17) and its use as a dietary supplement entails some expense and inconvenience. More importantly, CHL chemoprevention is not without some potential risk. Promotion by a post-initiation CHL exposure has previously been reported in the rat where 0.1% CHL in the drinking water for 20 weeks after initiation increased the incidence of 1,2-dimethylhydrazine (DMH)-induced colon tumors from 10 to 47% (18). Post-initiation treatment with 0.001% CHL in the drinking water for 47 weeks also significantly increased colon tumor multiplicity in response to DMH (19), and the same CHL dose by water for 16 weeks after 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) initiation significantly increased colon aberrant crypt foci (ACF)/colon in the rat (20).

A potential alternative to CHL is natural Chl, which is without known toxicity in mammals. In the one study where post-initiation effects of Chl on colon carcinogenesis were examined, Chl suppressed rather than promoted azoxymethane- (a metabolite of DMH) and IQ-induced ACFs (20). An additional attraction is the potential to achieve protective doses of CHl without supplementation. Dietary Chl intake comparable with the 100 mg per meal (0.13 mmole per meal) daily doses of CHL administered in the human intervention trial is obtainable by moderate/high consumption of green vegetables. Spinach leaves, for example, average 0.12 mmole Chl per 100 g wet weight, so consumption of 100 g of spinach, or equivalent vegetable, per meal would approximate the above dose of CHL. However, in comparison with CHL, the chemopreventive properties of natural Chl have received little study. While Chl is potently anti-mutagenic (reviewed in 21), and was recently shown to induce mammalian phase 2 detoxifying enzymes in vitro (22), only a few studies have suggested that natural Chl might have cancer preventive properties in whole animals. In the rainbow trout model of dietary carcinogenesis, hepatic DNA-adduct formation resulting from 200 ppm dibenzo[a,l]pyrene exposure was reduced 66% by co-exposure to 3000 ppm Chl in the diet (23). Moreover, the adduct inhibition by Chl was nearly identical to the protection afforded by a similar dietary co-exposure to CHL (23). In the rat colon, dietary spinach or an equimolar amount of Chl equally inhibited cytotoxicity and colonic crypt proliferation induced...

Abbreviations: ACF, aberrant crypt focus; AFB1, aflatoxin B1; ANOVA, analysis of variance; Chl, chlorophyll; CHL, chlorophyllin; DMH, 1,2-dimethylhydrazine; GST, glutathione S-transferase; GST-P, glutathione S-transferase placental form-positive foci; HCC, hepatocellular carcinoma; IQ, 2-amino-3-methylimidazo[4,5-f]quinoline; NQO, NAD(P)H:quinone oxidoreductase; TEG, triethylene glycol.
by heme, a red meat, iron-containing pro-oxidant correlated with increased risk of colon cancer (24,25). In addition, the Chl-containing diet largely blocked formation of a cytotoxic heme metabolite (24). The authors speculated that green vegetables may decrease colon cancer risk from dietary heme through the protective effects of Chl.

Although these studies suggest a potential role for dietary Chl in cancer prevention, there are at present no animal data demonstrating its efficacy. The present study used the male F344 rat gavage model of AFB1 carcinogenesis (4,5) to compare the effects of Chl and CHL co-exposure against early biomarkers of genome damage including AFB1–DNA adduction, serum albumin adduction and urinary AFB–N7-guanine excretion. We also examined AFB1 interactions with these two agents in vitro, as well as the in vivo response of hepatic phase 2 enzymes to Chl and CHL alone and in co-gavage with AFB1. A second study compared the effects of Chl and CHL on AFB1-induced pre-neoplastic lesions in the liver and colon. The results demonstrate that Chl and CHL have similar potency to reduce induced pre-neoplastic lesions in the liver and colon. The results are discussed below in the context of the mechanism(s) by which Chl may influence the expression of phase II enzymes in vitro and in vivo.

Materials and methods

Materials

CHL, AFB1, tricaprylin and triethylene glycol (TEG) were from Sigma Chemical Co. (St Louis, MO). [3H]-AFB1 (21.8 Ci/m mole) was from Moravek Biochemicals (Brea, CA). The purity and concentration of AFB1 was confirmed by absorbance in ethanol at 362 nm (ε362 = 2.18 × 10^5 M^-1 cm^-1). The chlorin content of CHL was based on the manufacturer’s assay of 4.5% copper and assertion that all copper was present as copper chlorins (Lot # 14H0002, 51.3% Cu chlorin content, additional material is water soluble Na salts). Highly purified Chl was prepared as described below.

Purification of Chl

A comprehensive description of the Chl purification will be presented elsewhere (C.Jubert and G.S.Bailey, in preparation). Briefly, organic spinach was washed with cold water, freeze-dried, washed twice with petroleum ether (3:1, v/v). The combined extracts were washed with saturated sodium chloride, filtered and evaporated in vacuo (temperature < 30°C). This crude Chl extract (90% pure by high-performance liquid chromatography) was further purified by counter current chromatography using an Ito multilayer coil separator–extractor (P.C., Potomac, MD). Analyses of counter current chromatography fractions were performed by high-performance liquid chromatography, mass spectrometry and proton nuclear magnetic resonance spectroscopy. A minor impurity was detected at δ 5.40 consisting of 0.35 protons upon integration, and the only other extra peaks were the NMR solvent (δ 2.05) and the two signals due to H2O and deuterated water (δ 2.78 and 2.75). Purity was estimated to be >95% compared with Chl-a standards (Sigma Chemical Co.), which were 90–92% pure based on spectroscopic measurements. Thirty grams of freeze-dried spinach yielded 225 mg of Chl-a and 75 mg of Chl-b for a total yield of 300 mg Chl, or 1% by dry weight. Chl preparations used in all experiments were a recombined 3:1 mixture of Chl-a:Chl-b.

Preparation of test solutions

Concentrated stocks (>1 mg/ml) of AFB1 were first prepared in dichloromethane and diluted to working concentrations in ethanol prior to dilution in the tricaprylin gavage vehicle. Final ethanol concentration in gavage solutions was <1% (v/v). Concentrated solutions of CHL were prepared in water and diluted to the administered concentration in tricaprylin gavage vehicle. Chl is virtually insoluble in water; thus, Chl solutions were prepared in petroleum ether, diluted to a working stock in ethanol and diluted to the administered concentration in tricaprylin gavage vehicle. Gavage solutions were monophasic with the exception of the AFB1–CHL co-gavage in which the non-chlorin salt fraction was insoluble (see Materials and methods).

Animals and diets

For the adduct study, male F344 rats, 75–100 g (Simonsen Laboratories, Gilroy, CA), were housed individually in metabolism cages, for easy collection of separate urines and feces, and maintained at 22°C on a 12-h light–dark cycle. For the liver and colon foci study, male F344 rats, 75–100 g, were housed two per cage in standard barrier cages (see details below). Rats were acclimated to AIN-93G diet (Dyets, Bethlehem, PA) without ethoxyquin antioxidant and fed ad libitum for 1 week prior to the period of carcinogen exposure.

Adduct study

After 1 week on the acclimation diet, the rats were randomly assigned to one of three treatment groups. The experimental treatments are summarized in Table I. Group 1 (N = 7) received by gavage 250 μg of [3H]-AFB1 (260 μCi/μmol) per kg of body wt (0.208 μCi/g body wt) in 3 μl of tricaprylin per gram of body wt. Group 2 (N = 7) received the same [3H]-AFB1 dose mixed with 250 mg CHL (as Cu chlorins) per kg body wt. Group 3 (N = 7) received the same [3H]-AFB1 dose mixed with 300 mg Chl per kg body wt. The amount of Chl administered was equimolar with the amount of CHL administered and, based on in vitro binding measurements, was calculated to complex at least 90% of the AFB1 in the gavage solutions at 25°C. All seven rats per group were gavaged on days 0, 1 and 2. Two hours after the day 2 gavage, four rats from each group were euthanized for tissue collection. The remaining three rats per group were gavaged on days 3 and 4, and 2 h after the day 4 gavage the remaining rats were euthanized for tissue collection. Rats were weighed daily throughout the experiment.

Isolation of DNA and analysis of hepatic aflatoxin–DNA adducts

Rats were euthanized with CO2 on the indicated days 2 h after gavage dosing. The liver was excised, cut into pieces of ~100 mg, which were divided equally between two 50 ml centrifuge tubes and immediately frozen in liquid N2 for later use. Approximately 1.2 g of frozen liver pieces were placed
in 20 volumes of DNAzol reagent (Molecular Research Center, Cincinnati, OH) and homogenized with a few pulses of an Ultra-Turrax T8 homogenizer (IKA Works, Wilmington, NC). DNA extraction using DNAzol was according to the manufacturer’s protocol, and quantification of DNA yield was done with PicoGreen reagent (Molecular Probes, Eugene, OR). A known mass of purified DNA (average 1 mg) from each liver was directly counted in a Beckman 7500 liquid scintillation counter as a measure of total DNA (signal/noise ratio >100 for all samples).

Quantification of aflatoxin—serum albumin adducts
Blood (~1 ml) was collected from each rat on gavage day 2 or 4 at necropsy. The blood was centrifuged at 300g for 5 min and the resultant serum was removed and stored at ~8°C until analysis. The serum albumin was purified by the method of Chapot and Wild (26) and quantified using Bradford dye reagent and albumin standards (Bio-Rad Laboratories, Hercules, CA). The samples were prepared for liquid scintillation counting by mixing 0.5 ml of purified albumin sample (1–2 mg albumin) with 1 ml Soluene 350 tissue solubilizer (Perkin Elmer, Shelton, CT) in a glass scintillation vial and heating the mixture at 55°C for 1 h. After cooling, 10 ml of Hionic Fluor LSC fluid (Perkin Elmer) was added to the solubilized samples and total AFB1 equivalents were determined radiometrically in a Beckman 7500 liquid scintillation counter (signal/noise ratio >100 for all samples).

Urinary AFB–N7-guanine and fecal AFB equivalents
Twenty-four hour urine samples were normalized using a spectrophotometric creatinine kit (TECO Diagnostics, Anaheim, CA). Urine aflatoxin metabolites were recovered using an aflatoxin-specific preparative monoclonal antibody immunooaffinity column as described previously (27–29). Metabolites were identified and quantified by electrospray mass spectrometry as described (30). Twenty-four hour fecal samples, ~1 g, were weighed and homogenized in 10 volumes of water, and 0.5 ml aliquots were decolorized overnight at room temperature with 30% hydrogen peroxide. A 0.1 ml aliquot of each sample (2 mg feces) was mixed with 10 ml of Hionic Fluor LSC fluid and counted in a Beckman 7500 liquid scintillation counter (signal/noise ratio >100 for all samples).

AFB1 interactions with CHL and Chl in vitro
Formation of a non-covalent complex between AFB1 and CHL or highly purified Chl was assessed by quenching of AFB1 fluorescence as described previously in detail for quenching of AFB2 (AFB2 structural analogue) fluorescence by CHL (31). Dissociation constant (Kd) determinations could not be carried out in the tricaprylin gavage solvent due to the strong fluorescence of that compound. We instead used a close approximation to tricaprylin, triethyleneglycol (TEG, pH 8.0), an amphiphatic solvent that provided appropriate linearity of AFB1 fluorescence and sensitivity for monitoring quenching. TEG was not used for gavage co-solvent due to its dehydrating effect on rats. The initial concentration of AFB1 (substrate) was 10 μM in a 3 ml stirred quartz cuvette. CHL or Chl was added in 1.33 μM increments up to 31.92 μM CHL or Chl, with negligible increase in assay volume from ligand additions. Fluorescence was monitored at 428 ± 8 nm with excitation at 368 ± 8 nm, and was recorded 2 min after each ligand addition on a SLM 8000 photon counting spectrophotometer (SLM Aminco, Urbana, IL). The fluorescence quenching data were iteratively fitted to 1:1 and 2:1 AFB1:CHL or Chl models of binding stoichiometry as described in (31).

Phase 2 enzyme responses
Two additional treatment groups indicated in Table I (N = 2 rats each) were gavaged concurrently with the rats in the adduct study and were used to measure phase 2 responses due solely to CHL or Chl exposure. These groups were gavaged on days 0–2 only, with 250 mg/kg CHL (as Cu chlorins) in tricaprylin or with 300 mg/kg Chl in tricaprylin. Approximately 0.5 g of frozen rat liver from each rat in the adduct study was homogenized in nine volumes of 3 mM Tris–HCl, pH 7.4, with 0.25 M sucrose and 1 mM ethyleneglycol-bis[aminoethyl]ether)-tetraacetic acid and then centrifuged at 100 000 g to yield microsomal-free cytosol. The cytosolic protein concentration of each sample was determined using the Bradford dye reagent with albumin standards. Cytosols were stored as 200 μl aliquots at ~8°C for enzyme assays. The Prochaska bioassay (32) was used to measure the activity of NAD(P)H:quinone oxidoreductase (NQO) using dilute cytosol in triplicate assays. Afterward, the rate of each reaction was normalized to the sample protein content as determined by crystal violet staining (32). The activity of glutathione S-transferase (GST) was similarly assayed, but at 340 mm at 25°C with 1-chloro-2,4-dinitrobenzene as the substrate. One milliliter of 0.1 M potassium phosphate, pH 6.6, reaction buffer contained 50 μl 20 mM glutathione, 50 μl 20 mM 1-chloro-2,4-dinitrobenzene (ethanolic stock) and dilute cytosol. The reaction rate was measured at 1 min intervals for 5 min on a Beckman Coulter DU800 spectrophotometer.

Late pathophysiological marker study
Forty-two male F344 rats (mean weight 140 g) were arranged into six treatment groups (Table I). Groups 1–3 (N = 10 rats each) were experimental groups, and groups 4–6 (N = 5, 4 and 3 rats each, respectively) were controls. Group 1 received 250 μg/kg body...
Chl and CHL effects on AFB1-DNA adduction in vivo

The effects of CHL and Chl on hepatic AFB1-DNA adduction in rats that received 250 μg/kg AFB1 per kg by gavage for 5 days are shown in Figure 2 (panel A). Liver samples were taken on two separate days at 2 h post-dosing, the time of maximal AFB–N7-guanine levels in the rat liver (38). The overall DNA adduction level, measured as [3H]-AFB1 equivalents, among the three treatments was slightly higher on day 2 than day 4. This is consistent with previous studies in which the maximum levels of carcinogen binding to hepatic DNA are observed following the first few doses of AFB1, and decline thereafter, despite continued exposure (6,39). Co-gavage with 250 mg/kg CHL or the molar equivalent dose of 300 mg/kg Chl reduced hepatic DNA adduction by 33% (P = 0.003) and 47% (P = 0.001), respectively, relative to the control group by day 2. At 2 h after the day 4 gavage, the control group adduct level had declined somewhat from day 2, but protection by CHL and Chl remained substantial, with adducts decreased by 42% (P = 0.031) and 55% (P = 0.008), respectively, relative to the control group.

Chl and CHL effects on AFB1-serum albumin adduction

Serum albumin is the major blood protein to be adducted following metabolic epoxidation of AFB1 (5,40,41). The levels of aflatoxin–serum albumin adduction were measured radiometrically and the impact of CHL and Chl on albumin binding is shown in Figure 2 (panel B). The temporal pattern of albumin-adduct formation and the degree of chemoprotection is similar to that seen in the DNA. Day 2 showed an overall higher level of adduction and both the CHL and Chl cotreatments significantly reduced adducts by 52% (P = 0.038) and 62% (P = 0.022), respectively, relative to the positive control group. On day 4, as we observed for DNA adduction, the degree of protection by CHL and Chl remained significant, with 65% (P = 0.001) and 71% (P < 0.001) respective reductions in albumin adducts. Also, as seen with DNA adducts, CHL and Chl protection on day 4 appeared slightly greater than on day 2, though not significantly so.
Chl and CHL effects on urinary and fecal elimination of aflatoxin metabolites

Previous studies have shown that, in mammals, the major AFB–N7-guanine adduct is rapidly excised and excreted via the urine (42,43), and that CHL-impeded absorption of AFB1 results in lower levels of excised DNA adducts in the urine, and correspondingly higher AFB1 equivalents in the feces (7). Table II shows the mean levels of the AFB–N7-guaine adduct and two major less-toxic aflatoxin metabolites, AFM1, and AFB1, in 24 h urine samples taken on day 4 (N = 3 rats per treatment). Statistical comparisons were made on natural log-transformed data and reported on the original (e⁰) scale. The level of the AFB–N7-guaine adduct excreted in the urine was significantly reduced by 90% (P = 0.0007) and 92% (P = 0.0029) by co-gavage with CHL and Chl, respectively, compared with the control group. AFM1 in the urine was significantly reduced by 63% (P = 0.0173) and 81% (P = 0.0016) by CHL and Chl, respectively. AFB1 excretion to the urine was also significantly reduced by 90% (P = 0.0003) and 92% (P = 0.0002) by CHL and Chl, respectively. The relative amounts of protection by CHL and Chl against the urinary AFB1–DNA repair product and AFM1 metabolite were identical. CHL and Chl appeared slightly less effective in reducing the level of AFM1 in urine, but the basis for this is unclear.

Conversely, the day 4, 24 h feces from the CHL and Chl co-gavaged rats contained 137% (P = 0.0003) and 412% (P = 0.0048) more AFB1 equivalents, respectively, than did feces of the control group (Figure 3). The fecal elimination of AFB1 equivalents in the CHL-co-treated rats was 2-fold greater (P = 0.0212) than the elimination by CHL-co-treated rats, a larger difference between CHL and Chl effects than seen with any other parameter except urinary AFM1 excretion.

Chl and CHL interactions with AFB1 in vitro

We previously reported that CHL formed a strong non-covalent 1:1 complex with AFB1 and AFB2 (8,9 position saturated analogue) in vitro (Kd = 1.4 and 1.92 μM, respectively) (13,31). Importantly, those studies were consistent with a higher affinity of CHL in vivo than that demonstrated in vitro. We considered the possibility of CHL and CHL impeding aflatoxin absorption (15) in vivo. The aflatoxin absorption trial showed a 2.2 ± 1.1% (P = 0.0003) and 412% (P = 0.0002) reduction in the absorption of CHL and Chl, respectively. It is possible that CHL and CHL impeded aflatoxin absorption via the gastrointestinal tract in vivo.

Table II. Effect of CHL on AFB1 metabolites in rat urine

<table>
<thead>
<tr>
<th>Gavage treatment</th>
<th>Metabolites (pmol/mg creatinine)</th>
<th>(control−treatment) and (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AFB1–N7-guaine</td>
<td>AFM1</td>
</tr>
<tr>
<td>250 μg/kg AFB1</td>
<td>259.1 ± 108.1</td>
<td>1467.4 ± 458.0</td>
</tr>
<tr>
<td>250 μg/kg AFB1 + 250 mg/kg CHL</td>
<td>20.8 ± 2.84</td>
<td>489.8 ± 30.0</td>
</tr>
<tr>
<td>250 μg/kg AFB1 + 300 mg/kg Chl</td>
<td>17.0 ± 3.3</td>
<td>261.2 ± 30.2</td>
</tr>
</tbody>
</table>

Statistically significant differences from the control are indicated with one or more asterisks: *P < 0.05, **P < 0.005 and ***P < 0.0005.

The multiplicative difference [e(control−treatment)] between control and treatment mean of the logged data: i.e. AFB1 alone produced 10.1 times and 12.65 times more AFB1–N7-guaine adducts than did co-treatment with CHL and Chl, respectively. The 95% confidence intervals (CIs) on the multiplicative differences are in brackets.

Discussion

The present studies demonstrate substantial protection by natural Chl against AFB1 carcinogenesis in the rat liver and colon. Hepatic DNA
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and the ethyl ester component (CD = 20 μM) is 50 times more potent an NQO inducer in vitro than Chl (22). Our NQO results with CHL co-exposure may reflect the inherent gap between cell culture and whole animal studies. Alternatively, differences in the dose and timing of CHL administration may account for different phase 2 induction. Dingley et al. (50) showed that rats given dietary CHL (10 g/kg diet) for 2 weeks prior to a single low-dose co-gavage of two heterocyclic amine carcinogens had a 1.6-fold increase in NQO activity relative to control animals 24 h after the carcinogen dose, but no change in GST activity. There are, however, substantial protocol differences in that study (a 40-fold higher concentration of CHL in the diet, administration of CHL for 2 weeks prior to the carcinogen) that might account for the difference in NQO induction. A whole animal study comparing multiple doses of CHL and Chl and pre- versus co-initiation protocols may help shed light on the relative importance of phase 2 metabolism in CHL and Chl chemoprevention.

Both CHL and Chl given co-initiation (during carcinogen exposure) were quite effective in reducing putative pre-neoplastic foci in the colon and the liver. Consumption of chemoprotectants by humans is likely to involve these agents acting on previous as well as concurrent initiation events. Thus, the post-initiation effects of CHL and Chl are important. Our companion study in the rainbow trout model (M.T. Simonich et al., in preparation) included examination of the co- versus post-initiation effect of 2000 ppm dietary CHL on dietary dibenz[a]pyrene-initiated tumors in multiple organs. Post-initiation CHL for 9 months significantly promoted swim bladder tumor incidence from 10 to 38%, and promotion approached significance in the stomach. Co-initiation CHL had no effect in the swim bladder, but significantly inhibited tumor incidence by 30% in the liver and stomach. In the rat, post-initiation treatment with 0.1% CHL in the drinking water for 20 weeks reportedly increased the incidence of DMH-induced colon tumors from 10 to 47% (18). In another study, post-initiation treatment of rats with 0.001, 0.01 or 0.1% CHL in the drinking water for 47 weeks significantly increased the multiplicity of DMH-initiated colon tumors only at the 0.001% CHL dose (19). In that same study, the same CHL regimen inhibited in a dose-related manner dietary IQ-initiated liver tumors, but had no effect on IQ-initiated colon tumors (19). A follow-up study to (19) reported that 0.001% CHL in the drinking water for 16 weeks after IQ gavage significantly increased IQ-induced ACFs (20). Importantly, that study also included an experiment that showed that 0.08% dietary natural Chl (approximately equivalent to 0.1% in the drinking water) inhibited azoxymethane- (a metabolite of DMH) and IQ-induced ACFs (20). Collectively, the pattern of post-initiation effects of CHL and Chl appears to depend heavily on several factors including concentration and duration of post-initiation exposure and the initiating carcinogen. Future work with Chl must carefully examine these factors to evaluate its true utility as a chemopreventive agent.

The present study provides limited evidence that Chl inhibits uptake of AFB1 from the rat stomach, and that it does so with equal or greater efficacy than CHL. In pharmacokinetic compartments outside the gastrointestinal tract such as the liver, serum and urine, the adduct burden was reduced over a 2- to 13-fold range by Chl co-exposure, whereas in the feces of the same animals, roughly 5-fold more AFB1 equivalents were eliminated relative to the control animals. Thus, Chl co-exposure largely restricted AFB1 to the gastrointestinal tract. Moreover, substantial protection by Chl against ACF development in the colon suggests that, in addition to restricting AFB1 to the gastrointestinal tract, Chl treatment reduced AFB1 metabolism to toxic intermediates in the colon. One simple mechanism to explain inhibition of both uptake and colon metabolism of the carcinogen could be formation of a molecular complex between CHL and AFB1, some heterocyclic amine carcinogens and dibenz[a]pyrene is easily demonstrated in vitro (13–15) using both fluorescence and absorbance spectrophotometry. We show here that Chl is also able to form an AFB1 complex of ~2.5 times greater stability in vitro. Interestingly, the chemoprotection afforded by Chl against AFB1-induced DNA damage, serum...
all groups. Only day 2 tissues were collected from the CHL and Chl control groups from
fluorescence units to recorded from the above spectra. Data were normalized by converting
the AFB1 emission spectrum from 380 to 550 (±8) nm (excitation 368 ± 8
l
l
250
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Groups significantly different than the AFB1 control group are indicated with an asterisk (*P < 0.05 and **P < 0.005).

Table III. Effect of Chl on hepatic phase 2 enzyme activities

<table>
<thead>
<tr>
<th>Gavage treatment</th>
<th>Activity (nmol/min/mg)</th>
<th>GST</th>
<th>NQO</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 2</td>
<td>Day 4</td>
</tr>
<tr>
<td>250 µg/kg AFB1</td>
<td>294.5 ± 17.60</td>
<td>332.2 ± 26.67</td>
<td>26.7 ± 4.81</td>
</tr>
<tr>
<td>250 µg/kg AFB1 + 250 mg/kg CHL</td>
<td>328.0 ± 8.63</td>
<td>282.2 ± 7.83*</td>
<td>28.3 ± 2.06</td>
</tr>
<tr>
<td>250 µg/kg AFB1 + 300 mg/kg Chl</td>
<td>250.0 ± 4.60</td>
<td>261.6 ± 6.29**</td>
<td>27.3 ± 1.69</td>
</tr>
<tr>
<td>250 mg/kg CHL</td>
<td>313.8 ± 9.80</td>
<td>24.6 ± 1.42</td>
<td>25.1 ± 5.58</td>
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<tr>
<td>300 mg/kg Chl</td>
<td>271.2 ± 10.83</td>
<td>22.2 ± 1.24</td>
<td>27.2 ± 2.81</td>
</tr>
</tbody>
</table>

*Liver tissue samples were collected as described in the legend to Figure 1. The day 2 and day 4 responses are mean responses ± standard errors for N = 4 animals per treatment on day 2 and N = 3 animals per treatment on day 4 (exception for Day 2 NQO activity, N = 3 for AFB1 treatment). Assays were done in triplicate for all groups. Only day 2 tissues were collected from the CHL and Chl control groups from N = 2 animals each.

**GST activity in 100 000 g cytosol was assayed once per minute for 5 min at 25°C as described in Materials and methods. NQO activity in 100 000 g cytosol was assayed at the end of a 5 min reaction at 25°C as described.

Fig. 4. Spectrofluorometric titration of AFB1 with Chl. (A) Effect of Chl on the AFB1 emission spectrum from 380 to 550 (±8) nm (excitation 368 ± 8 nm) with AFB1 (substrate) concentration at 10 µM. Chl (ligand) was added in 1.33 µM increments up to 31.92 µM (some titrations omitted from the figure for clarity) and the spectrum was recorded 2 min after each addition. (B) Quantification of Chl quenching of AFB1 fluorescence at 428 ± 8 nm recorded from the above spectra. Data were normalized by converting fluorescence units to AF/FO and the data were fitted to a 1:1 Chl:AFB1 complexation model.
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Acknowledgements

We especially thank Mandy Louderback for her excellence in rat husbandry and necropsy, and Adam Sinner for his diligent work on the enzyme assays as part of his training project. We thank Dr Michael Schimelk of the Department of Biochemistry and Biophysics for help with modeling the fluorescence binding data. We also thank Yonghai Li and Cody Olsen of the Department of Statistics for their help with data analyses. Partly supported through National Institutes of Health grants CA900890, ES00210, ES03850, CA100608 and CA65525.

Conflict of Interest Statement: None declared.

References


Fig. 5. Effects of CHL and Chl on the hepatic burden of AFB₁-induced GST-P-positive foci and the colonic burden of ACFs. Rats were gavaged five times per week for 2 weeks as described in Materials and methods, killed 18 weeks after the last gavage and the livers and colons were processed as described. Samples from both organs were coded and scored blind. (A) Volume percent of the liver occupied by GST-P foci. (B) ACFs per colon. Values in (A and B) are the means ± standard errors for N = 9, 10 and 10 rats in experimental groups 1–3, respectively. The vehicle, CHL and Chl-negative control groups had N = 5, 4 and 3 rats, respectively. The probability value (P) for treatment difference from the AFB₁ control group is indicated above each treatment bar. P < 0.05 was considered significantly different.

Received July 31, 2006; revised January 4, 2007; accepted January 29, 2007