Inhibition of dibenzo[a,l]pyrene-induced multi-organ carcinogenesis by dietary chlorophyllin in rainbow trout

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Cancer chemoprevention by dietary chlorophyllin (CHL) was investigated in a rainbow trout multi-organ tumor model. In study 1, duplicate groups of 130 juvenile trout were treated for 2 weeks with control diet, 500 p.p.m. dibenzo[a,l]pyrene (DB[a,l]P) or 500 p.p.m. DB[a,l]P + 2052 p.p.m. CHL, then returned to control diet. DB[a,l]P alone proved somewhat toxic but induced high tumor incidences in liver (61%), stomach (91%) and swimbladder (53%) 11 months after initiation. CHL co-feeding abrogated incidences in liver (61%), stomach (91%) and swimbladder (P ≤ 0.01). A second tumor and DNA adduct study using a non-toxic initiation protocol (200 p.p.m. DB[a,l]P ± 4000 p.p.m. CHL for 4 weeks) confirmed these results. Potential CHL inhibitory mechanisms were investigated. Dietary CHL inhibited hepatic DB[a,l]P-DNA adducts in the two tumor studies by 89 and 76%, respectively. CHL co-feeding abrogated DB[a,l]P acute toxicity and reduced tumor incidences to 18% in liver, 34% in stomach and 3% in swimbladder (P ≤ 0.01). A second tumor and DNA adduct study using a non-toxic initiation protocol (200 p.p.m. DB[a,l]P ± 4000 p.p.m. CHL for 4 weeks) confirmed these results. Potential CHL inhibitory mechanisms were investigated. Dietary CHL inhibited hepatic DB[a,l]P-DNA adducts in the two tumor studies by 89 and 76%, respectively. CHL was shown to complex strongly with DB[a,l]P (Kd1,2 = 1.59 ± 0.01 μM, stoichiometry 2CHL:DB[a,l]P) and strongly inhibited DB[a,l]P mutagenesis in the Salmonella assay. Significant inhibition occurred at CHL concentrations substantially less than stoichiometric with DB[a,l]P and thus not reflecting simple DB[a,l]P sequestration via complexation. These initial findings suggest that CHL chemoprevention reflects complexation that might limit DB[a,l]P uptake in vivo, antimutagenic mechanisms such as catalytic degradation of the proximate electrophile in target cells, or both. These results demonstrate that dietary CHL is a reproducibly effective chemopreventive agent for DB[a,l]P multi-organ tumorigenesis in trout and suggest that reduced DB[a,l]P-DNA adducts may be predictive biomarkers of CHL reduction of DB[a,l]P-initiated hepatic tumors.

Introduction

Epidemiological data indicate that a high consumption of fruits and green vegetables is associated with reduced risk for some cancers (1,2). Despite the consistency of this observation, the protective factors in fruits and vegetables responsible for reduction of human cancer risk have not been clearly established. However, numerous candidate chemical constituents purified from fruits and vegetables have been shown to protect against carcinogens in experimental animal models (3–5) and a few have progressed to clinical trials. Among the latter category, chlorophylls are of particular interest owing to their ubiquity in green plants and potential for a simple chemoprotective mechanism involving reduced carcinogen bioavailability in vivo. Chlorophyllin (CHL), a water-soluble sodium/copper derivative of natural chlorophylls, has been used without apparent toxic side effects to treat a number of human conditions, including control of body, fecal and urinary odor of geriatric patients (6,7) and treatment of calcium oxalate stone disease (8,9). Rodent studies also suggest efficacy in wound healing (10). CHL and chlorophyll are potent antimutagens against a wide range of potential human carcinogens (11–15). Recently, CHL was shown to inhibit the genotoxic and carcinogenic activity of aflatoxin B1 (AFB1) in rainbow trout (16). Subsequent studies also revealed efficacy against the tumorigenicity of 2-amino-3-methylimidazo[4,5-f]quinoline (17–19), 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) (20,21), benzo[a]pyrene and 7,12-dimethylbenz[a]anthracene (DMBA) (22,23) in rodent models.

These results suggest that CHL may have chemoprotective activity against a variety of carcinogens, especially those having at least partially planar aromatic or polyaromatic structures. Dibenz[a]pyrene (DB[a,l]P) is the most potent carcinogen known among polycyclic aromatic hydrocarbons. This planar polyaromatic hydrocarbon is a potent tumor initiator in mouse skin and rat mammary gland (24–26) and mouse lung (27) and a multi-organ carcinogen in the rainbow trout (28). Though its carcinogenic risk to humans has not been fully assessed, DB[a,l]P has been detected in cigarette smoke condensate (29), soil and sediment samples (30) and in particulates formed by combustion of low sulfur content coal (smokey coal) (31). Chemopreventive agents such as 4-hydroxymercuribenzoate (32) and chlorophylls (33,34) have been shown to reduce DB[a,l]P-DNA adduction, but inhibition of DB[a,l]P-induced tumorigenesis by CHL has not been reported in any model. The rainbow trout model has proven to be a sensitive, statistically powerful vertebrate alternative for comparative studies of chemical carcinogenesis and its modulation by dietary inhibitors, promoters and co-carcinogens (35). The present study employed this model to investigate the effects of dietary CHL co-treatment on hepatic DNA adduction and multi-organ tumorigenesis by DB[a,l]P. The results provide the first evidence for a CHL-mediated reduction in DB[a,l]P multi-organ tumorigenesis and suggest that hepatic DNA adducts may be appropriate biomarkers for predicting aspects of risk reduction.

Materials and methods

Abbreviations: AFB1, aflatoxin B1; BNF, β-naphthoflavone; CHL, chlorophyllin; DB[a,l]P, dibenzo[a,l]pyrene; DMBA, 7,12-dimethylbenz[a]anthracene; DMSO, dimethylsulfoxide; DTE, dithioerythritol; OTD, Oregon test diet; PhIP, 2-amino-1-methyl-6-phenylimidazo-[4,5-b]pyridine; THF, tetrahydrofuran.

Materials

DB[a,l]P was obtained from the NCI Chemical Carcinogen Reference Standard Repository (Kansas City, MO). For fluorescence experiments DB[a,l]P was
dissolved in spectrophotometric grade, inhibitor-free tetrahydrofuran (THF) (Aldrich, Milwaukee, WI) and stored under argon. Concentrations of DB[a]P in THF solution were verified spectrophotometrically, based on absorbance at 317 nm (in THF solution were verified spectrophotometrically, based on absorbance at 317 nm (∊317 = 5.14 × 10^4). For HPLC and mutagenicity assays, DB[a]P was dissolved in dimethylsulfoxide (DMSO). All solutions were protected from light and stored at −4°C when not in use. For tumor studies the DB[a]P was dissolved in the oil component of the trout semi-synthetic Oregon Test Diet (OTD) formula (36). (Note: DB[a]P is a potent carcinogenic compound; it was handled, stored and disposed of in compliance with NIH and Oregon State University guidelines for class C carcinogens.) CHL was obtained from Sigma (St Louis, MO). Since commercially available CHL is a mixture of sodium/copper chlorophyllin and several inorganic salts, all CHL solution and dietary concentrations for in vitro experiments were corrected to the actual copper chlorophyllin content (51.3%) of the lot used in these studies. The remaining constituents of the Sigma CHL sample used were, according to the supplier, inorganic salts, primarily NaCl. Since the basic trout diet formulation already contains 40 000 p.p.m. ionizable salts, including NaCl, an additional 1000–2000 p.p.m. NaCl from the Sigma CHL formulation is expected to have negligible effect on tumor response. CHL in solution has the property of self-association, resulting in a time-dependent reduction in effective concentration. Thus, for each in vitro experiment CHL was dissolved in the appropriate buffer immediately prior to use. For tumor studies CHL was dissolved in the water component of the OTD formulation. Since both CHL and DB[a]P are light-sensitive compounds, all diets and solutions containing either compound were protected from exposure to light and experiments were conducted under subdued lighting. Diets were prepared every 2 weeks and stored in the dark at 4°C. The stabilities of DB[a]P and CHL in the diet were not directly investigated in this study, however, separate experiments have demonstrated 90–100% recovery of DB[a]P from trout diet stored at −20°C for up to 2 years (unpublished data).

Tumor studies

Study 1. Shasta strain rainbow trout (Oncorhynchus mykiss) were fed control OTD from swim-up to 18 weeks of age. Beginning at 18 weeks, duplicate tanks of 130 fish each were prefixed the control diet for one additional week (groups 1 and 2) or OTD containing 2052 p.p.m. CHL (as total copper chlorin) on a dry diet basis (group 3). This was followed for the next 2 weeks with a diet containing 500 p.p.m. DB[a]P alone (group 2), 2052 p.p.m. CHL + 500 p.p.m. DB[a]P (group 3) or OTD only (group 1). DB[a]P concentrations were also on a dry diet basis. Three groups of 5 fish/tank were removed 2 days after DB[a]P treatment ceased and killed to obtain livers for DNA adduct analysis. This protocol left an initial 115 fish/tank for growth- and tumor development. Final numbers killed in Table 1 are less than this and represent losses due to normal mortalities and to acute toxicity.

The 500 p.p.m. DB[a]P 2 week initiation protocol in study 1 proved to be above the maximum tolerated dose in our Shasta trout, inducing an excess mortality rate at the end of the carcinogen treatment period and consequent elevated growth rate among the remaining survivors in group 2 fed this dose of DB[a]P alone (Table 1). To correct for the confounding effect of body weight differences on tumor outcome, final body weight was included as a variable in logistic regression modeling as described previously (37). In addition, we conducted a second experiment (study 2) at a reduced carcinogen dose (200 p.p.m.) for an extended exposure period (4 weeks) to avoid acute toxicity leading to mortality.

Study 2. Duplicate tanks with 130 fingerling rainbow trout of 20 weeks age were prefixed the control OTD diet (group 1) or OTD containing 4000 p.p.m. CHL on a dry diet basis (group 2). This was followed by 4 weeks of feeding OTD containing 200 p.p.m. DB[a]P alone (group 1) or 200 p.p.m. DB[a]P + 4000 p.p.m. CHL (group 2). Reduced DB[a]P dose was expected to yield lower tumor response in study 2. Therefore, CHL concentration was raised to 4000 p.p.m. in order to optimize probability of observing statistically significant reduction in tumor response. Three groups of 6 fish/tank were removed from each tank at the end of the 4 week treatment period and killed to obtain livers for DNA adduct analysis. Another 7 fish/tank were removed at this time for preliminary histochemical analyses, leaving 105 fish/tank for grow-out and tumor development. In contrast to study 1, no acute losses following DB[a]P treatment were observed in this experiment. We did not incorporate a 260 fish no treatment control into the experimental design. Instead, a negative control consisting of two tanks of 100 fish/tank was taken from an experiment run concurrently with study 2 under identical conditions. This experiment, which involved 8579 no treatment control fish, revealed a spontaneous background of 0.12% liver, 0.18% stomach and 0% swimbladder tumors under these experimental conditions. These background rates are consistent with those in study 1.

The remaining fish in both studies were fed OTD for the next 11 (study 1) or 9 (study 2) months until they were killed. At termination, livers, stomachs and swimbladders were removed from each fish and examined under a dissecting microscope for gross tumors (>0.5 mm diameter), fixed in Bouin’s solution and processed by routine histological methods. Historically, all stomach and swimbladder tumors and >95% of liver tumors in the trout are surface-oriented growths that are readily enumerated and measured at gross necropsy. One slide from each fish and each organ having one or more suspect tumors at necropsy was prepared for histology, so the histological analysis was not exhaustive. Tumors were classified according to criteria established previously by Hendricks et al. (38) for liver neoplasms and Hendricks et al. (39) and Bailey et al. (35) for stomach and swimbladder neoplasms. Percentages of the different histological types of liver neoplasms were based on the total number of each type divided by the total number of all hepatic neoplasms observed in that group. Tumor multiplicity or the average number of tumors/organ was calculated by dividing the total number of tumors observed by the number of tumor-bearing fish.

Table I. Modulation of DB[a]P tumorigenesis by dietary CHL in rainbow trout

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Initial fish no.</th>
<th>Final fish no.</th>
<th>Weight (g)</th>
<th>Tumor incidence (%)</th>
<th>Liver tumor types (%)</th>
<th>Tumor multiplicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>260</td>
<td>165</td>
<td>162 ± 48</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>500 p.p.m. DB[a]P</td>
<td>260</td>
<td>105</td>
<td>225 ± 64</td>
<td>61</td>
<td>91</td>
<td>53</td>
</tr>
<tr>
<td>500 p.p.m. DB[a]P + 2052 p.p.m. CHL</td>
<td>260</td>
<td>182</td>
<td>144 ± 44</td>
<td>18*</td>
<td>34*</td>
<td>3*</td>
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<td>Study 2</td>
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<tr>
<td>Control</td>
<td>200</td>
<td>174</td>
<td>97 ± 38</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>200 p.p.m. DB[a]P</td>
<td>260</td>
<td>177</td>
<td>115 ± 50</td>
<td>36</td>
<td>48</td>
<td>30</td>
</tr>
<tr>
<td>200 p.p.m. DB[a]P + 4000 p.p.m. CHL</td>
<td>260</td>
<td>175</td>
<td>103 ± 44</td>
<td>13*</td>
<td>13*</td>
<td>5*</td>
</tr>
</tbody>
</table>

Note:

- a One year tumor incidences. Data from treatments with duplicate tanks have been combined. Thirty fish from each dose were removed on day 23 for DNA adduct analysis. Groups significantly different from the DB[a]P-alone group are shown by an asterisk (*P < 0.001).
- b Relative percentage of each tumor type compared with the total tumors observed. HCC, hepatocellular carcinoma; MC, mixed hepatocellular/cholangiocarcinoma; HCA, hepatocellular adenoma; CCA, cholangiocarcinoma adenoma.
- c Mean tumors per tumor-bearing fish (SD values omitted to conserve space). Groups significantly different from the DB[a]P-alone group are shown by an asterisk (*P < 0.01).
- d Concurrent control (see Materials and methods).

DNA adduction

DNA was isolated from trout liver using phenol and chloroform extractions, precipitated with ethanol and dissolved in Tris-EDTA buffer. Concentration and purity were estimated using UV absorbance at 260 and 280 nm. Control and adducted liver DNA (10 or 25 μg) was digested, labeled and analyzed by both 32P-post-labeling and 33P-post-labeling HPLC essentially as described by Gupta et al. (40) and Ralston et al. (41), respectively.

DB[a]P–CHL complexation

Standardization experiments indicated that a buffer of 20% THF in 0.1 M Tris at pH 7.4 provided appropriate CHL and DB[a]P solubility, linearity of DB[a]P fluorescence and ample sensitivity for assessing CHL quenching.
CHL chemoprevention in trout organs

(data not shown). To establish the quenching effect of CHL on the DB[a,l]P fluorescence spectrum, emissions from a 1.48 µM solution of DB[a,l]P in 20% THF buffer were measured from 400 to 500 nm at an excitation of 317 ± 8 nm at 25°C. Spectra were recorded after each 1.0 µM incremental increase in CHL concentration, up to a final concentration of 5 µM, in reaction volumes of 3 ml. In the buffered 20% THF, DB[a,l]P fluorescence response began to lose linearity at concentrations >1.2 µM. Thus experiments to quantify DB[a,l]P-CHL complex formation used a DB[a,l]P concentration of 1.18 µM in order to maximize assay sensitivity within the linear range. To establish CHL–DB[a,l]P complex stoichiometry and dissociation constants, fluorescence was measured at 425 ± 8 nm under excitation at 317 ± 8 nm, at final CHL concentrations ranging from 0.0 to 5.0 µM in 0.2 µM increments. At least two determinations were made at each of three replicates for each CHL concentration.

Modeling of CHL–DB[a,l]P complex formation

CHL–DB[a,l]P complex formation was modeled by taking the resulting data set from above and using an iterative modeling process. Data were first modeled assuming a 1:1 CHL to DB[a,l]P stoichiometry, however, fitting of the data to this model was poor and the non-random nature of the residuals [successive differences between ΔF/Fo data points and the fitted curve (Ft)] did not support a 1:1 complex stoichiometry (modeling plots not shown). A model for a 2:1 ratio of CHL to DB[a,l]P binding was next constructed, as follows:

\[ K_{d1} \]
\[ 2 \text{CHL} + \text{DB[a,l]P} \rightleftharpoons K_{d2} \text{CHL} - \text{DB[a,l]P} \]

The dissociation constants \( K_{d1} \) and \( K_{d2} \) for this system are given as:

\[ K_{d1} = [\text{CHL}] [\text{DB[a,l]P}] / [\text{CHL} - \text{DB[a,l]P}] \]
\[ K_{d2} = [\text{CHL}] [\text{CHL} - \text{DB[a,l]P}] / [\text{CHL} - \text{DB[a,l]P}][\text{CHL}] \]

Fitting of the fluorescence quenching data to this model is presented in Results.

Preparation of CYP1A-induced trout liver microsomes

Trout hepatic CYP1A was induced by dietary β-naphthoflavone (BNF) treatment. Trout weighing ~25 g were fed OTD containing 500 p.p.m. BNF for 3 days, with one daily feeding of ~0.02 g diet/g trout. On the third day trout were killed by cervical spinal transection, their livers removed and frozen in liquid nitrogen and stored at −80°C until use. Pooled livers were homogenized in 4 vol of homogenization buffer [100 mM Tris, 100 mM KCl, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithioerythritol (DTE)] using a Potter–Elvejhem apparatus with a motor driven pestle. The homogenate was centrifuged for 20 min at 10 000 g in a pre-cooled (4°C) Beckman type 70 rotor. The supernatant was removed and further centrifuged for 90 min at 100 000 g. The resulting supernatant was discarded and the pellet was resuspended in 1 vol of resuspension buffer (100 mM potassium phosphate, pH 7.3, 1 mM EDTA, 20% glycerol, 1 mM DTE). Microsomal preparations were stored at −80°C. Microsomal protein content was determined by the Lowry method (42).

Induction of CYP1A enzymes was verified by measuring the rate of ethoxyresorufin O-deethylation in the presence of microsomes and NADPH as described (43).

Salmonella mutagenicity test

Salmonella typhimurium tester strain TA100 was kindly supplied by the B.N. Ames laboratory (University of California, Berkeley, CA). All media and genotype verifications for the Salmonella mutagenicity assays were as detailed by Maron and Ames (44). To increase the sensitivity of the assays, DB[a,l]P, CHL and TA100 were pre-incubated with BNF-induced trout microsomes in the presence of NADPH before plating. CYP1A-induced microsomes were chosen because initial experiments indicated poor DB[a,l]P metabolism by control trout microsomes. Each experiment included platings of pre-incubation mixture without DB[a,l]P to monitor spontaneous reversion rates. Assays were carried out at five CHL doses: 0, 0.1, 1, 10 and 50 nmol/ml, with at least three plates at each dose. Pre-incubations containing buffer (250 µM sucrose, 80 µM Tris, 25 mM KCl, 5 mM MgCl2, pH 7.4), 250 µg microsomal protein, 3 mM NADPH, varying CHL doses in the same buffer and 100 µl of fresh TA100 culture were initiated by the addition of 10 nmol DB[a,l]P in 10 µl DMSO. Total pre-incubation volumes were 250 µl. After 30 min at 25°C the pre-incubation mixture was transferred to a tube containing 2 ml of top agar (65°C agar) and 0.25 ml 0.5 mM histidine/methylthionic solution held at 45°C. Each tube was vortexed gently then poured onto minimal glucose medium plates. Following solidification of top agar the plates were inverted and incubated at 37°C in the dark for 48 h. Wild-type (his') revertants were counted manually.

Results

CHL effects on DB[a,l]P tumorigenicity

Overall tumor responses and CHL-mediated inhibition. As shown in Table I, study 1, a 500 p.p.m. dietary DB[a,l]P treatment of fingerling trout for 2 weeks was sufficient to induce a strong, multi-organ tumor response 11 months later. The primary target organ under the conditions tested was the stomach, followed by the liver and swimbladder. Stomach and swimbladder neoplasms were all benign papillary adenomas in all groups, as observed previously with other carcinogens (35,39). The variety of liver tumor phenotypes was also as previously observed (35–38) and included malignant and benign neoplasms of hepatocellular, cholangiocellular or mixed hepato/cholangiocellular origin. However, hepatocellular carcinoma was the predominant tumor phenotype initiated by DB[a,l]P, rather than the mixed carcinoma previously observed in trout using other carcinogens (45,46).

Co-exposure to dietary CHL strongly reduced the observed tumor response in all three target organs (Table I). CHL co-exposure also resulted in a significant (P < 0.01) reduction in tumor multiplicity in two of the three target organs and, in this study, an apparently higher ratio of benign hepatocellular adenomas to hepatocellular carcinomas.

Differential mortality and growth rates among treatment groups. No significant difference in mortality or final body weight was found between trout fed control diet (group 1) and DB[a,l]P + CHL diet (group 3) in study 1 (Table I). However, mean body weights and mortality rates of groups 1 and 3 were substantially lower than those of group 2 fed DB[a,l]P alone. The mortality rate in trout fed DB[a,l]P alone was 54%, compared with 28% in control and 21% in DB[a,l]P + CHL fed fish. Two conclusions are immediately apparent: (i) based on mortality alone, dietary CHL provided protection against DB[a,l]P acute toxicity; (ii) DB[a,l]P fed at 500 p.p.m. for 2 weeks exceeded the maximum tolerated dose to trout of the age and size used in this study. The higher mean final body weight among survivors in group 2 may be attributed to a growth-promoting effect associated with lower fish density and perhaps to a founder effect (i.e. possible selective survival of bigger fish of the toxic effects of 500 p.p.m. DB[a,l]P).

As a consequence, the above calculations of CHL anti-tumorigenic efficacy and the mechanistic implications of those calculations are clearly subject to the confounding effect of growth difference between groups 2 and 3 of study 1. Two approaches were taken to address this issue. First, owing to low husbandry costs sufficient trout are routinely used in tumor studies that the potentially confounding effects of growth difference on tumor response can be quantified, and possibly removed, by regression modeling (37). This analysis, shown in Figure 1, is based on partitioning of individual animals for each treatment group into equal quintiles with respect to mean final growth achieved and regression of growth quintiles within each group against final tumor incidence observed among individuals within that quintile. As previously reported for DMBA-initiated trout (37), tumor response in all three target organs in study 1 is indeed positively correlated with final growth achieved (i.e. the curves have non-zero slopes, P < 0.001). In addition, the analysis shows that CHL co-treatment significantly inhibited tumor response in all three target organs, independently of greater growth seen in group 2, i.e. if the reduced incidences in group 3 were due solely to
a lower mean population growth rate relative to group 2, then the two data sets for each organ would necessarily describe a single line in which the group 3 data points collect at the lower weight end of that line. Regression of the data sets for groups 2 and 3, however, yields lines that are not co-linear but differ significantly ($P < 0.001$) for all three target organs.

Thus regression analysis demonstrates significant CHL protective effects independent of growth differences among treatment groups. Of equal importance, it is also possible to calculate the magnitude of CHL protection relative to the pseudo-protection due to excess growth in the positive control group. To assess this, we note for example that group 2 displayed 91% stomach tumor incidence at a mean weight of 225 g. The regression equation for group 2 is then used to calculate the tumor incidence that would be expected at a mean weight of 225 g. The estimation of 82% incidence, rather than the 34% incidence actually observed in group 3. By similar calculations, the lower mean growth in the CHL-alone treatment group would have reduced liver incidence from 61% to 44% and swimbladder incidence from 53% to 39%, rather than the observed 18% and 3%, respectively. Based on the differences between growth-predicted and observed final incidences, we can estimate that CHL treatment per se accounted for a reduction of 60%, 84% and 78% of the incidence change seen for liver, stomach and swimbladder, respectively, with the remaining change due to elevated mean population growth in group 2.

As a second approach, we have conducted an entirely independent tumor study that confirms the ability of CHL to provide strong anticarcinogenic protection independent of any growth-related mechanism. This study employed a 4 week, 200 p.p.m. DB[a,l]P initiation protocol, which we had established in a 42 000 trout DB[a,l]P tumor study to induce no acute toxicity or growth differences from the untreated control group (unpublished data). The study design also incorporated a higher CHL concentration than was used in study 1. As seen in Table I, study 2, this protocol induced lower overall tumor incidence in the DB[a,l]P-only group, but, as expected, produced no acute toxicity. No growth rate differences were observed between treatment groups in study 2. However, as in study 1, co-treatment with CHL produced comparable inhibition of tumor incidence in all three target organs (e.g. absolute incidence reductions of 23–35%, 73–89% inhibition of tumor odds). Also as in study 1, CHL co-exposure resulted in a significant reduction in tumor multiplicity in stomach and swimbladder ($P < 0.01$), but not in liver. A CHL-only control group was not included in either study because previous experiments (16) have shown that dietary CHL up to 4000 p.p.m. for 9 months does not alter spontaneous tumor incidence rates, induce toxicity, alter hepatosomatic index or impair growth.

**CHL effects on hepatic DB[a,l]P–DNA adduction**

Analysis of trout liver DB[a,l]P–DNA adducts by 32P-post-labeling showed that DB[a,l]P formed several stable adducts (Figure 2), with five major and several minor adducts detectable. Although the identities of individual trout DB[a,l]P–DNA adducts remain to be established, total stable adducts were readily quantifiable. CHL treatment reduced the formation of total liver DB[a,l]P–DNA adducts by 89% in study 1 (Table II). TLC resolution, especially at the higher exposure of the DB[a,l]P group, did not justify individual adduct quantification. However, studies using superior 33P-post-labeling HPLC resolution among adducts provided no evidence for substantial change in the ratios among the major adducts (data not shown). The 33P-post-labeling HPLC method was also used to quantify DNA adduct formation in study 2. We note that the absolute levels of DNA adduction in the two studies cannot be directly compared owing to the use of different exposure and adduct measurement protocols. However, the results clearly show that CHL co-exposure strongly inhibited carcinogen–DNA adduction in study 2 (76%), as it did in study 1. Methods adequate to reliably quantify DB[a,l]P–DNA adducts in trout stomach and swimbladder were not available for this study and the present results apply only to hepatic DNA adducts.

**CHL–DB[a,l]P complex formation in vitro**

This experiment addressed the possibility that DB[a,l]P may form a non-covalent complex with CHL of sufficient stability to limit procarcinogen systemic bioavailability or target organ metabolic activation. CHL quenching of DB[a,l]P fluorescence emission would reflect radiationless energy transfer from the excited state of the fluorescing species to the ground state of the proximate quenching molecule and thus can be taken as an indirect measure of complex formation (47,48). As seen in Figure 3A, titration of DB[a,l]P with CHL (under conditions of negligible CHL fluorescence or quenching of the excitation energy) resulted in a uniform quenching of the DB[a,l]P fluorescence emission spectrum between 400 and 500 nm. No peak shifts or changes in fluorescence characteristics other than quenching were observed in this experiment. The next experiment quantified CHL quenching by making 0.2 μM incremental additions of CHL to a 1.18 μM DB[a,l]P solution and measuring the fractional fluorescence change ($\Delta F/F_0$) at any growth-related mechanism. This study employed a 4 week, 200 p.p.m. DB[a,l]P initiation protocol, which we had established in a 42 000 trout DB[a,l]P tumor study to induce no acute toxicity or growth differences from the untreated control group (unpublished data). The study design also incorporated a higher CHL concentration than was used in study 1. As seen in Table I, study 2, this protocol induced lower overall tumor incidence in the DB[a,l]P-only group, but, as expected, produced no acute toxicity. No growth rate differences were observed between treatment groups in study 2. However, as in study 1, co-treatment with CHL produced comparable inhibition of tumor incidence in all three target organs (e.g. absolute incidence reductions of 23–35%, 73–89% inhibition of tumor odds). Also as in study 1, CHL co-exposure resulted in a significant reduction in tumor multiplicity in stomach and swimbladder ($P < 0.01$), but not in liver. A CHL-only control group was not included in either study because previous experiments (16) have shown that dietary CHL up to 4000 p.p.m. for 9 months does not alter spontaneous tumor incidence rates, induce toxicity, alter hepatosomatic index or impair growth.

**CHL effects on hepatic DB[a,l]P–DNA adduction**

Analysis of trout liver DB[a,l]P–DNA adducts by 32P-post-labeling showed that DB[a,l]P formed several stable adducts (Figure 2), with five major and several minor adducts detectable. Although the identities of individual trout DB[a,l]P–DNA adducts remain to be established, total stable adducts were readily quantifiable. CHL treatment reduced the formation of total liver DB[a,l]P–DNA adducts by 89% in study 1 (Table II). TLC resolution, especially at the higher exposure of the DB[a,l]P group, did not justify individual adduct quantification. However, studies using superior 33P-post-labeling HPLC resolution among adducts provided no evidence for substantial change in the ratios among the major adducts (data not shown). The 33P-post-labeling HPLC method was also used to quantify DNA adduct formation in study 2. We note that the absolute levels of DNA adduction in the two studies cannot be directly compared owing to the use of different exposure and adduct measurement protocols. However, the results clearly show that CHL co-exposure strongly inhibited carcinogen–DNA adduction in study 2 (76%), as it did in study 1. Methods adequate to reliably quantify DB[a,l]P–DNA adducts in trout stomach and swimbladder were not available for this study and the present results apply only to hepatic DNA adducts.

**CHL–DB[a,l]P complex formation in vitro**

This experiment addressed the possibility that DB[a,l]P may form a non-covalent complex with CHL of sufficient stability to limit procarcinogen systemic bioavailability or target organ metabolic activation. CHL quenching of DB[a,l]P fluorescence emission would reflect radiationless energy transfer from the excited state of the fluorescing species to the ground state of the proximate quenching molecule and thus can be taken as an indirect measure of complex formation (47,48). As seen in Figure 3A, titration of DB[a,l]P with CHL (under conditions of negligible CHL fluorescence or quenching of the excitation energy) resulted in a uniform quenching of the DB[a,l]P fluorescence emission spectrum between 400 and 500 nm. No peak shifts or changes in fluorescence characteristics other than quenching were observed in this experiment. The next experiment quantified CHL quenching by making 0.2 μM incremental additions of CHL to a 1.18 μM DB[a,l]P solution and measuring the fractional fluorescence change ($\Delta F/F_0$) at
allowed for possible quenching differences between the inter-molecule upon complexation with CHL, whereas a 2:1 model resulted in a poor fit of the data to the model. A model for a 1:1 ratio of CHL to DB[a]P, assumed to mediate (1:1) and final (2:1) complexes. The total fluorescence ($F_{\text{tot}}$) of the system is then given by:

$$F_{\text{tot}} = F[DB[a]P] + p_1 F[CHL-DB[a]P] + p_2 F[CHL_2-DB[a]P]$$

where $p_1$ and $p_2$ are the fluorescence enhancements for the complexes CHL–DB[a]P and CHL_2–DB[a]P, respectively, and $F$ is the molar fluorescence of DB[a]P. Fitting of the data set to the 2:1 model is shown in Figure 3B. Residuals for this model (data not shown) were small and without apparent trend, indicating a good fit of the data to the modeled curve.

The derived fitted values for $K_{d1}$ and $K_{d2}$ depended on the assumptions made concerning the values of $p_1$ and $p_2$. The simplest model assumes that the fluorescence ‘enhancement’ for each complex is equal to 0 ($p_1 = p_2 = 0$) and also assumes that the two dissociation constants are equal ($K_{d1} = K_{d2}$). For this model the curves showed a very good fit ($r^2 = 0.9997$) and gave a single value of $K_{d1} = 1.59 \mu M$ ($\sigma = 0.01$). The system was also modeled to allow differential $K_d$ values and fluorescence enhancements for the two complexes. For example, a model with $p_1 = 0.185$ and $p_2 = 0$ gave a very good fit ($r^2 = 0.9998$) and inferred dissociation constants of $K_{d1} = 1.29 \mu M$ ($\sigma = 0.03$) and $K_{d2} = 1.01 \mu M$ ($\sigma = 0.04$). Other parameter variations under this second scheme also provided satisfactory fits, yielding dissociation constants ranging between 1.0 and 1.9 \mu M. We accept these as the limiting values for either of the two dissociation constants for the (CHL)_2–DB[a]P complex and the single value of 1.59 \mu M as a reasonable measure of overall complex stability.

**CHL effects on DB[a]P mutagenesis**

This experiment considered the possibility that CHL may, by any of several possible mechanisms, inhibit the microsome-catalyzed conversion of parent DB[a]P to bacterial mutagens.

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**Table II. Inhibition by dietary CHL of DB[a]P adduction to liver DNA in vivo**

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Adducts (nmol/mol DNA)$^a$</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study 1$^b$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>657 (± 128.5)</td>
<td></td>
</tr>
<tr>
<td>500 p.p.m. DB[a]P</td>
<td>69.5 (± 7.5)</td>
<td>89</td>
</tr>
<tr>
<td>Study 2$^b$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>200 p.p.m. DB[a]P</td>
<td>5500 (± 960)</td>
<td></td>
</tr>
<tr>
<td>200 p.p.m. DB[a]P + 4000 p.p.m. CHL</td>
<td>1330 (± 340)</td>
<td>76</td>
</tr>
</tbody>
</table>

$^a$Numbers are means (± range) of duplicate pools from two tanks. Adducts were quantified by 32P-post-labeling TLC and represent total adducts.

$^b$Numbers are means (± SD) of six replicate pools from two tanks. Adducts were quantified by 32P-post-labeling HPLC and represent total adducts.
As shown in Table III, CHL was a potent antimutagen against DB[a,l]P. In particular, a significant decrease in mutagenicity was seen with the addition of sub-stoichiometric quantities of CHL relative to DB[a,l]P in this assay. Based on mean values, >50% inhibition of mutagenesis was seen at a CHL concentration only 10% that of DB[a,l]P (P < 0.001) and some evidence for inhibition was seen even at 10-fold lower CHL (P = 0.076), i.e. when DB[a,l]P exceeded CHL by 100-fold. Inhibition at such low molar ratios of CHL to DB[a,l]P demonstrates that simple sequestration of promutagen via complex formation between the two compounds cannot be the major mechanism responsible for CHL antimutagenesis in vitro in these experiments, CHL is known to exhibit concentration-dependent inhibition of CYP1A ethoxyresorufin O-deethylation (49) and thus may be interfering directly with CYP1A-mediated DB[a,l]P metabolism. However, this mechanism has not been directly tested for trout CYP1A-mediated DB[a,l]P metabolism. A second possibility, which also remains to be tested, is that CHL may catalyze degradation of DB[a,l]P or its proximate electrophile, as it does benzo[a]pyrene diol epoxide (49).

**Discussion**

### CHL chemoprevention of DB[a,l]P tumorigenesis in the trout model

The present studies show that CHL is highly effective at inhibiting multi-organ carcinogenesis in trout by dietary co-exposure with DB[a,l]P. In two separate studies, dietary co-treatment with CHL strongly reduced tumor incidence in liver, stomach and swimbladder and also significantly reduced tumor multiplicity in the latter two organs. There are several quantitative indices of CHL chemopreventive efficacy and consideration of these can provide mechanistic insight in a multi-organ setting. For example, the commonly applied calculation of percent inhibition of tumor incidence (100P<sub>CHL</sub>/P<sub>0</sub>), where P is the proportion of tumor-bearing animals) would suggest that CHL inhibition in study 1 (Table I) was greatest in swimbladder (94%), followed by the liver (71%) and stomach (63%). This apparent difference in protection among target organs would thus suggest organ-specific CHL inhibitory mechanisms superimposed on, or in place of, any systemically global preventive mechanism. We have recently determined, however, that this calculation can provide comparisons that are highly dependent on carcinogen dose examined and hence potentially mechanistically misleading (C.Pereira and G.S.Bailey, unpublished results). In contrast, calculations based on inhibition of tumor odds (proportion of tumor-bearing to tumor-free individuals, P/[1 – P]) can provide a metric of inhibition that is independent of carcinogen dose or incidence in the positive control. When applied to the data in study 1, the calculated percent inhibition of tumor odds are 86, 92 and 97% in liver, stomach and swimbladder, respectively. This measure of inhibitor efficacy suggests a more uniform CHL-mediated inhibition of tumor response among all target organs. This impression of uniformity is supported by an alternative measure, which is that co-treatment with CHL provided an absolute tumor incidence reduction near 50% for all three target organs in study 1.

Although tumor incidence was a useful end-point for assessing multi-organ CHL efficacy in this study, multiplicity was not, i.e. CHL co-treatment reduced multiplicity as well as incidence in stomach and swimbladder, but reduced incidence alone in liver. The basis for this differential effect, in particular the relationship between carcinogen dose, CHL dose, tumor incidence and tumor multiplicity, has not yet been established in this model. Present results suggest that these relationships will differ among the three target organs.

### Mechanisms of CHL chemoprevention

The mechanistic basis for CHL anticarcinogenic activity is not completely understood and may well vary with the model studied. Among the mechanisms previously proposed are molecular trapping by complex formation with parent mutagens or activated intermediates, scavenging of radicals and active oxygen species, inhibition of metabolic activation enzymes and induction of phase II enzymes (15,23,49–53). We note that dietary CHL does not detectably alter hepatic levels of CYP1A, glutathione transferase or UDP-glucuronosyl transferase in the trout (54). Evidence is presented herein that CHL can exhibit an antimutagenic activity against DB[a,l]P in vitro at a stoichiometry which suggests mechanisms other than complex-mediated DB[a,l]P sequestration (trapping). Possible mechanisms include electrophile scavenging or cytochrome P450 enzyme inhibition. However, further research is needed to establish if either of these mechanisms may apply for DB[a,l]P in vitro and to establish whether in vivo target organ and organelle CHL levels following dietary treatment are sufficient to sustain any such localized, cellular mechanism. We note that such mechanisms would seem likely to be variable among target organs according to localized CHL concentrations and cellular metabolic characteristics and would not likely provide uniform protection in all target organs.

An alternative mechanism with potential for systemically uniform protection involves complex formation with CHL resulting in reduced carcinogen uptake and bioavailability. As noted above, CHL co-treatment protected against tumor response in all three trout organs to approximately the same extent when measured by absolute incidence reduction (50 ± 7% observed range incidence reduction in study 1; 29 ± 6% incidence reduction in study 2) or percent inhibition of tumor odds (92 ± 5% in study 1; 81 ± 8% in study 2). The in vitro complexation experiments described herein indicate formation of a 2:1 CHL–DB[a,l]P complex with dissociation constants K<sub>1</sub> and K<sub>2</sub> estimated at 1.59 ± 0.01 μM, if assumed equal, or ranging between 1.0 and 1.9 μM with other parameters modeled. These values are very similar to the K<sub>4</sub> of 1.4 ± 0.4 μM for the AFB<sub>1</sub>–CHL complex (53), formation

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**Table III. Inhibition by CHL of DB[a,l]P mutagenicity in Salmonella typhimurium tester strain TA-100**

<table>
<thead>
<tr>
<th>CHL (nmol/plate)</th>
<th>Revertants&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Inhibition&lt;sup&gt;c&lt;/sup&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>373 ± 43</td>
<td>0</td>
</tr>
<tr>
<td>0.1</td>
<td>338 ± 14&lt;sup&gt;d&lt;/sup&gt;</td>
<td>15</td>
</tr>
<tr>
<td>1.0</td>
<td>249 ± 24&lt;sup&gt;d&lt;/sup&gt;</td>
<td>52</td>
</tr>
<tr>
<td>10</td>
<td>211 ± 28&lt;sup&gt;d&lt;/sup&gt;</td>
<td>67</td>
</tr>
<tr>
<td>50</td>
<td>117 ± 18&lt;sup&gt;e&lt;/sup&gt;</td>
<td>106</td>
</tr>
</tbody>
</table>

<sup>a</sup>Tester strain, CHL and DB[a,l]P (10 nmol/plate) were preincubated with BNF-induced trout liver microsomes for 30 min at 25°C.

<sup>b</sup>Mean ± SD of at least three replicate plates (n = 7, 6, 3, 6 and 8 for CHL doses of 0–50 nmol, respectively).

<sup>c</sup>Percent inhibition after subtraction of background revertants (132 ± 7).

<sup>d</sup>Difference from 0 CHL positive control not significant (P = 0.076, two-sided t-test).

<sup>e</sup>Significantly different from 0 CHL control (P < 0.001).
of which has been shown to greatly reduce systemic AFB¹ uptake and biodistribution (54,55), target organ DNA adduction (15,16) and tumor initiation (16). As a consequence, it may provide an assembly of similar size and stability to the AFB¹–CHL complex and, presumably, with similarly reduced bioavailability from the gastrointestinal tract in vivo. Pharmacokinetic studies to investigate CHL effects on DB[a]P bioavailability will be needed to more directly investigate this hypothesized mechanism.

**CHL chemoprevention in other species**

Considerable evidence is accumulating that CHL can be considered a blocking agent against a variety of genotoxic carcinogens and in a variety of animal models. In addition to its efficacy in trout against DB[a]P and AFB¹, Guo et al. (19) have demonstrated inhibition of multi-organ carcinogenesis in male F344 rats by CHL. CHL (1% in diet) administered simultaneously with PhIP significantly reduced mammary adenocarcinomas in female F344 rats (56). Park and Surh (22) have shown that oral administration of CHL protected against the tumorigenicity of topically applied benzo[a]pyrene and its metabolite benzo[a]pyrene diol epoxide in mouse skin. In contrast, however, it must be noted that CHL has been reported to promote rather than protect against colon carcinogenesis in the rat dimethylhydrazine model (57) and there was evidence for decreased tumor latency in heterocyclic amine-induced skin tumorigenesis (19). Mechanisms responsible for these negative attributes of CHL and the basis for the apparently opposing effects of CHL in the PhIP and dimethylhydrazine rat carcinogenesis models are not presently understood.

**Conclusions**

The present study demonstrates a substantial chemoprotective effect of CHL against multi-organ carcinogenesis by DB[a]P in trout when given by dietary co-exposure. Although the first study led to an unexpected excess mortality and excess weight gain in the carcinogen-only control, it was possible to clearly demonstrate CHL reduction in tumor initiation independent of treatment-related differences in population mean growth rate in this experiment and to confirm this by a second tumor study devoid of acute toxicity or differential growth complications. We have shown that CHL significantly reduced DNA adduction in at least one target organ, liver, at the end of the carcinogen exposure period, as well as final tumor incidence, which indicates that DNA adducts are at least qualitatively predictive biomarkers of final tumor reduction in this target organ. However, a quantitative molecular dosimetry study such as was carried out previously for CHL and AFB¹ (16) will be necessary to establish if reduced DB[a]P–DNA adducts in liver will serve as biomarkers that precisely predict reduced hepatic tumor risk for this carcinogen. The effects of CHL on DB[a]P–DNA adduction and their usefulness as biomarkers of risk reduction in stomach and swimbladder remain to be established.

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