The importance of carcinogen dose in chemoprevention studies: quantitative interrelationships between, dibenzo[a,l]pyrene dose, chlorophyllin dose, target organ DNA adduct biomarkers and final tumor outcome

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Chlorophyllin (CHL) is a potent antimutagen in vitro, an effective anti-carcinogen in several animal models, and significantly reduced urinary biomarkers of aflatoxin B1 (AFB1) exposure in a human population. Here we report an expanded analysis of CHL chemoprevention using the potent environmental hydrocarbon dibenzo[a,l]pyrene (DBP). A dose–dose matrix design employed over 12,000 rainbow trout to evaluate the interrelationships among dietary carcinogen dose, anti-carcinogen dose, carcinogen–DNA adduct levels at exposure and eventual tumor outcome in two target organs. Included was an evaluation of the pharmaceutical CHL preparation (Derifil), used previously in a study of individuals chronically exposed to AFB1. CHL was pre-, co- and post-fed at doses of 0–6000 p.p.m. and co-fed with DBP at doses of 0–371.5 p.p.m. for 4 weeks. This protocol generated a total of 21 dose–dose treatment groups, each evaluated with three or more replicates of 100 animals. The DBP-only treatment produced dose-responsive increases in liver and stomach DBP–DNA adducts, whereas increasing CHL cotreatment doses produced successive inhibition in liver (49–83%) and stomach (47–75%) adduct levels at each DBP dose examined. The remaining 8711 trout were necropsied, 10 months later. DBP treatment alone produced a logit incidence versus log [DBP] dose–response curve in stomach that was linear; CHL co-treatment provided dose-dependent tumor inhibition which ranged from 30 to 68% and was predictable from the adduct response. The Derifil CHL preparation was also found to effectively reduce DNAadduction and final tumor incidence in stomach (as well as liver), with a potency compatible with its total chlorin content. Liver tumor incidence in the DBP-only groups appeared to plateau near 60%. At DBP doses of ≤80 p.p.m., increasing CHL doses generally reduced tumor incidence and multiplicity consistent with early DNA adducts as biomarkers. At 225 p.p.m. DBP, however, very high CHL doses were required to reduce tumor incidence below the 60% plateau. Apparent tumor multiplicity in liver was neither linear nor monotonic with DBP dose, but peaked at 80 p.p.m. DBP and declined at 225 p.p.m., where it was increased by all but one CHL dose. Consequently, the effects of a given CHL dose and the predictivity of DNA adducts as biomarkers were highly dependent on carcinogen dose. These results underscore the critical importance of establishing carcinogen-end point dose–response relationships in chemoprevention studies, and the potential otherwise for misleading interpretations in chemoprevention studies carried out solely at high-carcinogen dose. (Supported by USPHS grants ES03850, ES00210, CA34732, ES07060, ES06052 and ES03819.)

Introduction

Carcinogens can be found in numerous sources, including industrial pollution, ultraviolet light, tobacco products and the diet. It has been estimated that 35% of cancer deaths in USA can be attributed to dietary imbalances, which may include an insufficiency of protective dietary factors, as well as the presence of dietary carcinogens (1). The antimutagenic and anticarcinogenic properties of numerous compounds found in the human diet have been demonstrated using in vitro and animal model systems (2–4). Prospective studies designed to validate a relationship between diet and cancer risk in humans have yielded mixed results (5,6). However, data from case–control studies suggest that cancer risk may be reduced by altering diet and/or lifestyle (7–9).

Research over the past decade has shown that chlorophyllin (CHL), a widely available derivative of natural chlorophyll, can substantially reduce tumor response in a variety of animal models and target organs. CHL has been used by human populations for over 50 years for medicinal purposes with no reported adverse effects (10–15). As reviewed by Waters et al. (16), CHL is a very effective inhibitor of numerous mutagens, including aflatoxin B1 (AFB1), polycyclic aromatic hydrocarbons (PAHs), heterocyclic amines, direct-acting compounds and complex mixtures. When given orally, CHL is a potent agent for the reduction of DNA binding resulting from oral 2-amino-3-methylimidazo [4,5-f]quinoline (IQ) and 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP) treatments in rats (17,18). The anticarcinogenic properties of this promising agent were first demonstrated in the rainbow trout model, in which dietary co-treatment produced dose-responsive inhibition of
initial AFB1–DNA adduct biomarkers to an extent that predicted reduction in eventual tumor outcome (19). In subsequent studies, CHL administered in the drinking water of male F344 rats delayed or reduced liver, Zymbals gland, small, and large intestine tumorigenesis initiated by IQ gavage (20) and in female F344 rats, CHL given in the diet along with PhIP resulted in a reduction in mammary adeno- carcinoma multiplicity (21). Topical application of CHL in a murine skin model reduced 7,12-dimethylbenz[a]anthracene (DMBA)-initiated papillomagenesis (22) and antitumor effects of CHL have been reported against diethylnitosamine-phenobarbital-induced male F344 rat hepatocarcinogenesis (23) and against DMBA–TPA (12-O-tetradecanoylphorbol-13-acetate)-induced mouse skin carcinogenesis (24). Clinically, a double-blinded, placebo-controlled study was undertaken in AFB1-endemic areas of China where urinary AFB1–N7-guanine DNA adduct repair products were validated as biomarkers of AFB1 exposure (25). Taken orally in the amount of 100 mg at meal time three times per day, CHL reduced the recovery of these biomarkers by >50% (26), an effect comparable to the protection against AFB1 seen earlier in trout (19).

CHL has also proven effective against environmental PAHs. Human dietary exposure to PAHs has been estimated at 3 μg/day for non-smokers, an exposure comparable to the estimated 2–5 μg PAH/pack of cigarettes (27). PAHs can be formed on foods during the cooking process or can be deposited on foods as smoke condensate (28). This is a particular problem in areas such as rural China where unvented stoves are used for indoor cooking and heating (29). The inhabitants of these regions experience high rates of lung (30) and esophageal cancer (31). Esophageal biopsy samples taken from individuals in this region have yielded detectable PAH–DNA adducts (32). Dibenzo- pyrenes have been identified in airborne particulates collected from urban areas (33). The dibenzo[a,l]pyrene (DBP) isomer has specifically been identified as a combustion product in coal smoke (30) and tobacco smoke (34). Though present in small quantities, this compound is a potent mutagen (35,36). DBP is also a potent carcinogen in dietary fish models (37,38) and has been described as the strongest PAH carcinogen ever tested in mouse skin models (39,40). DBP has consistently been assigned toxicity equivalent factor values ranging from equivalency with to well in excess of benzo[a]pyrene, indicating that DBP is considered an extremely potent carcinogen regardless of methodology used (28).

We have previously demonstrated in an initial single-dose study that dietary co-treatment with CHL could substantially reduce tumor response in multiple target organs in rainbow trout fed DBP (37). The purpose of the present report is to extend these findings, using a more complete dose–dose matrix design to evaluate the complex interrelationships among dietary DBP dose delivered, dietary CHL dose delivered, initial target organ DBP–DNA adducts as biomarkers of effective exposure and ultimate tumor outcome. The rainbow trout models used in these experiments express a wide range of cytochrome P450 metabolizing enzymes (41), bioactivate many procarcinogens including DBP (37,42) and have a well-established pathology (43,44). A principal attribute of this model is its low husbandry costs, which allows for statistically complex designs requiring large numbers of animals not generally affordable with rodent models (44). The present dose–dose matrix design employed an initial 12,350 animals in 21 DBP–CHL dose–dose combinations plus two additional treatment groups to evaluate the CHL tablet (Derifil) and placebo formulations utilized in the clinical trial against AFB1 in China (26).

Materials and methods

DBP was obtained from the National Cancer Institute (NCI) Chemical Carcinogen Reference Standard Repository at Chemsys Laboratories (Lenexa, KS). Handling and storage of this potent carcinogen was in accordance with NIH and Oregon State University guidelines for Class C carcinogens. CHL, proteinase K, nuclease P1, prostatic acid phosphatase, apyrase, and snake venom phosphodiesterase were purchased from Sigma Chemical (St Louis, MO). T4 nucleotide kinase was purchased from United States Biochemical (Cleveland, OH). RNAase A and T1 cocktail was purchased from Ambion (Austin, TX). Radiolabeled ATP[γ-33P] was purchased from New England Nuclear (Boston, MA). Organic chemicals used were HPLC grade and were purchased from EM Science (Gibbstown, NJ).

The dose of Sigma CHL was based on the copper content stated on the label (lot 77H0594, Cu content 3.69%) and corrected to the actual copper chlorin content of 42.05%, as it was assumed that all Cu was bound to chlorins as CuCHL. The relative compositions of the major CHL constituents in the Sigma and Derifil preparations were determined following the HPLC method of Egner et al. (45). The Sigma preparation consisted of 72% chlorine ε6 and 10% chlorine ε4 (Figure 1). Derifil and placebo tablets were generous gifts of Rystan (Little Falls, NJ). The CHL content of the Derifil tablets was 100 mg per tablet, with a stated purity of 98% copper chlorins. The Derifil preparation was found to contain 51% chlorine ε6, 33% chlorine ε4 and 4% chlorine ε3 ethyl ester, the latter two being indentified in the serum of individuals consuming the Derifil tablets (45). As listed in the product insert, the ‘inactive ingredients’ in the Derifil tablets and the visually identical placebo tablets (26) consisted of ‘dextrose USP, hydrogenated vegetable oil NF, hydroxypropyl methylcellulose USP, magnesium stearate NF, micro-crystalline cellulose NF, peppermint powder, polyethylene glycol NF, sodium chloride USP.

DBP was dissolved in the oil component of the trout semi-synthetic Oregon Test Diet (OTD) (46). CHL, Derifil and placebo tablet powder were dissolved in the water portion of the diets. The concentrations of both DBP and CHL are expressed in parts per million (p.p.m.) relative to the dry weight portion of the diet. For those diets containing DBP or CHL or both, the oil and water containing specified amounts of DBP and CHL, respectively, were used in the same fashion as the oil and water portions of the diets containing neither DBP nor CHL. It should be noted that the OTD formulation sets to a cheese-like consistency that does not dissolve readily in water. Also, trout show rapid Pavlovian training to OTD exposure, such that all rations are consumed within seconds of deposition into the tank. In addition, each 100-gallon tank is under continuous water flow of ~2 gallons/min. Under these circumstances, aqueous exposure to carcinogens or other factors included in the OTD formulation is negligible, as is the opportunity for meaningful DBP–CHL interaction within the tank water. Both DBP and CHL are light-sensitive compounds and were handled, when possible, under subdued light conditions.
lighting or under lighting with a 400 nm cut-off. Diets were prepared every 2 weeks and stored at −20°C until 1 day prior to feeding, at which time they were removed to 4°C. A separate experiment examined DBP recovery from diets both with and without CHL following storage at −20°C for a period of up to 3 years (see below).

Animals

Shasta strain rainbow trout (Oncorhynchus mykiss) were spawned, raised and treated at the Food Toxicology and Nutrition Laboratory (FTNL), Oregon State University as described previously (44,46) under protocols meeting NIH guidelines and receiving IACUC approval. Fish fry (∼1.5 g/fish) were fed control OTD diet during the time between feeding onset and initial initiation. The fish were used from four separate groups of brood stock that were spawned on the same date. In total, 12,350 trout were selected and distributed, 130 fish per tank, to 95 tanks in random order. Each tank contained equal representation of fish from the four groups of brood stock. The fish were acclimated in their respective tanks for 1 week prior to the commencement of CHL prefeeding.

Dosage determination

The concentrations of DBP used were based, in part, on a previous study, which showed that 500 p.p.m. DBP exceeded a maximum tolerated dose, that dose was chosen as log (10.1, 28.4, 80.0 and 225 p.p.m.) or half-log (371.5 p.p.m.) intervals.

Study design

The dietary co-treatment protocol was chosen to mimic the intervention strategy used in the China human intervention trial (26), in which CHL was provided with each meal to test its ability to mitigate the effects of concurrent dietary carcinogen exposure. Post-initiation CHL effects were not included in this design because it is already known not to modulate hepatic tumor response in trout initiated with AFB1 (19) or DBP (Bailey et al., unpublished results) or in rats initiated with AFB1 (47). Post-initiation CHL will, at certain doses, suppress liver tumor response in rats initiated with a heterocyclic amine (48).

In the present study, CHL was pre-fed 1 week at 0, 1500, 3000, 4500 or 6000 p.p.m., then co-fed with the varying doses of DBP (see Table 1) for 4 weeks, followed by 1 week of post-initiation feeding with CHL, to assure CHL presence throughout DBP metabolism. Higher dose ranges of DBP were chosen as log (10.1, 28.4, 80.0 and 225 p.p.m.) or half-log (371.5 p.p.m.) intervals.

Isolation of hepatic and stomach DNA and determination of DBP–DNA adducts

Twenty nine days after commencement of carcinogen feeding, five fish were removed from each tank and euthanized. The livers and stomachs were removed and pooled by tank and organ to provide a minimum of N = 3 tank samples for each CHL–DBP treatment. All collected samples were quick-frozen in liquid nitrogen then stored at −80°C. DNA was purified from thawed, pooled liver samples and stored as described previously (37). Pooled stomachs were homogenized, digested with RNAse cocktail (Ambion) and proteinase K (100 μl at 20 μg/μl, pH 8, freshly prepared), extracted using NaCl and chloroform–isoamyl alcohol (50), then precipitated with ethanol, dissolved in TE and stored at −20°C.

The purified DNAs were post-labeled as described (51) with the omission of the dilute basic methanol rinse prior to elution with basic methanol.

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Samples were stored overnight at −20°C prior to enrichment of the DBP–DNA adducts using C-18 SepPak cartridges. Following washing of the SepPak cartridges to remove non-adducted nucleotides, adducts were eluted by the addition of precisely 3.0 ml of basic methanol (5% NH4OH) to the cartridge and 3.0 ml eluate was collected and 3 x 10 μl aliquots were taken from each sample for liquid scintillation counting (LSC) analysis to determine the total radioactivity contained in each sample. To determine what percentage of the radioactivity actually contained adducts, the remaining samples were concentrated to near dryness and reconstituted with ~50 μl, each, of deionized water and methanol to a nominal final volume of 100 μl. The samples were mixed thoroughly, transferred to autosampler vials, and placed in the sample compartment (maintained at 10°C) of the Waters 2690 HPLC. Analysis of the samples was performed using a C-18 column (Waters Symmetry 2.1 x 150 mm) in a 35°C, temperature-controlled compartment. A gradient elution was performed using methanol:acetonitrile (9:1; solvent A) and 0.1 M ammonium phosphate buffer (pH 9.1; solvent B) beginning at 20% A, with a constant flow rate of 0.4 ml/min. The mobile phase changed according to the following gradient: 0 min, 20% A; 12.5 min, 22.5% A; 17.5 min, 23.5% A; 45 min, 47.5% A; 62.5 min, 57.5% A; 72.5 min, 80% A; and 75 min, 20% A. Between samples, the column was equilibrated for 15 min. An inline Packard TR505 radio metric detector (0.5 ml cell, 1.6 ml/min scintillant) was used to detect the post-labeled adducts. Total adduct peak areas were combined with the LSC radioactivity measurements to quantify liver and stomach DBP–DNA adduct levels in the absence and presence of CHL. We note that statistical analysis of the post-labeled data revealed an unexplained but significant effect of post-labeling date on 1 of the 5 days in which liver adducts were analyzed. Therefore, samples evaluated on this particular date (all of the placebo replicates and some additional sample replicates) were omitted from the modeling, figures and Table II.

Determination of tumor response

Following sampling for DNA adduction, the remaining fish in each tank were fed OTD and allowed to grow for 3-12 additional months, at which time the fish were evaluated for tumor production. A planned, six tank pre-sacrifice sampling was conducted to evaluate 9 months after initiation to evaluate the extent of tumor formation at the highest doses and determine the timing of the final tumor sampling. The duration of final sampling of all 23 treatment groups was expected to be 8 weeks. A sampling schedule was devised so that the mean time of sampling over all tanks in each treatment group was 48 weeks post-initiation ± 1 day. Sampling was conducted in three blocks of time with each block containing a third of the tanks from each treatment. Within each block, tanks were sampled randomly with minor modifications to narrow the deviation to within ± 1 day of the mean for all treatments. In addition, the daily ration of OTD was reduced to maintenance rations to further reduce any time-related effect of fish growth on final tumor incidence. Retrospective analysis revealed no difference in tumor response between replicate treatment tanks sacrificed before and after the 48-week median date.

The fish were sacrificed by immersion in a lethal concentration of tricaine methanesulfonate in water, weighed individually and bled by cutting several gill arches. Body and liver weights were recorded, and livers and stomachs were examined under a dissecting microscope for the presence of suspect tumors. Swim bladders and kidneys were screened and inspected under the dissecting microscope if abnormalities were noted. (It has been observed that kidney tumors are surface oriented and readily detectable by visual inspection.) All grossly observable liver, stomach and swim bladder tumors were counted, and all organs with suspected tumors were fixed in a Bouin’s solution and processed by routine histological techniques. One slide from each organ having one or more suspect tumors, as determined by gross pathology, was prepared for histology. By this procedure, liver tumor incidence was based on confirmed neoplasms, whereas multiplicities included unconfirmed gross lesions as well. Confirmed neoplasms were classified according to the criteria established by Hendricks et al. (43,44). Tumor incidence was expressed as the percentage of fish bearing one or more confirmed tumors at each dose. Apparent tumor multiplicity, which includes gross data for the liver, is defined as mean number of gross tumors per organ for those animals having tumors. The tumor phenotype spectrum in liver was reported according to the percentage of each tumor phenotype found at that dose.

Analysis of stored diets

To evaluate the potential for DBP degradation when stored in OTD, both with and without CHL, a sub-sampling of diets stored for 3 years at −20°C was analyzed following the protocol of Loveland et al. (52). Four diets were analyzed, each in triplicate. The results demonstrated that DBP doses of 28.4 and 225 p.p.m. were recovered at near-targeted levels (98.3 and 98.0%,...
<table>
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<tr>
<th>[DBP] (p.p.m.)</th>
<th>[CHL] (p.p.m.)</th>
<th>No. of tanks</th>
<th>N</th>
<th>Tumor incidence&lt;sup&gt;a,b,c&lt;/sup&gt;</th>
<th>Mean body weight (g)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Liver somatic index&lt;sup&gt;a,d&lt;/sup&gt;</th>
<th>No. of liver tumors examined&lt;sup&gt;b,e&lt;/sup&gt;</th>
<th>% Liver tumors examined&lt;sup&gt;b,e&lt;/sup&gt;</th>
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<td>0</td>
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<td>0.69 (0.06)</td>
<td>9</td>
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<tr>
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<td>0.58 (0.02)</td>
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<td>192</td>
<td>15.1</td>
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</table>

<sup>a</sup>Mean (SE).

<sup>b</sup>---” indicates no tumors observed.

<sup>c</sup>“n.a.” indicates only one tank contained tumor-bearing fish.

<sup>d</sup>Somatic index is defined as mean liver weight (g)/mean body weight (g).

<sup>e</sup>HCC, hepatocellular carcinoma; HCA, hepatocellular adenoma; MC, mixed hepatocellular/cholangiocellular carcinoma; MA, mixed hepatocellular/cholangiocellular adenoma; CCC, cholangiocellular carcinoma; CCA, cholangiocellular adenoma.
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Table II. Liver and stomach DNA adduct response in trout fed varying doses of DBP and CHL

<table>
<thead>
<tr>
<th>[DBP] (p.p.m.)</th>
<th>[CHL] (p.p.m.)</th>
<th>Liver adducts (nmole adducts/mole DNA)abc</th>
<th>Stomach adducts (nmole adducts/mole DNA)ab</th>
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<tr>
<td></td>
<td></td>
<td>Total</td>
<td>% Polar</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>139.4 (16.5)</td>
<td>73.0 (2.9)</td>
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<td>1599.0 (67.8)</td>
<td>72.6 (1.6)</td>
</tr>
<tr>
<td>225</td>
<td>3000</td>
<td>464.5 (n.a.)</td>
<td>68.8 (4.2)</td>
</tr>
<tr>
<td>10.1</td>
<td>4500</td>
<td>3373.7 (87.1)</td>
<td>74.8 (9.3)</td>
</tr>
<tr>
<td>28.4</td>
<td>4500</td>
<td>1652.0 (209.2)</td>
<td>76.8 (3.3)</td>
</tr>
<tr>
<td>80</td>
<td>4500</td>
<td>2676.3 (n.a.)</td>
<td>69.6 (7.5)</td>
</tr>
<tr>
<td>225</td>
<td>4500</td>
<td>4818.0 (n.a.)</td>
<td>38.0 (19.3)</td>
</tr>
<tr>
<td>371.5</td>
<td>4500</td>
<td>6000.0 (228.7)</td>
<td>70.8 (7.6)</td>
</tr>
<tr>
<td>80</td>
<td>6000</td>
<td>13000.0 (228.7)</td>
<td>72.6 (1.6)</td>
</tr>
<tr>
<td>225</td>
<td>6000</td>
<td>2784.8 (n.a.)</td>
<td>72.1 (4.7)</td>
</tr>
<tr>
<td>371.5</td>
<td>6000</td>
<td>3799.7 (n.a.)</td>
<td>73.9 (3.6)</td>
</tr>
<tr>
<td>80</td>
<td>Derifil</td>
<td>615.3 (n.a.)</td>
<td>70.6 (1.5)</td>
</tr>
<tr>
<td>80</td>
<td>Placebo</td>
<td>NDd</td>
<td>60.5 (9.4)</td>
</tr>
</tbody>
</table>

abcMean (SE).

abAdduct levels not corrected for background.

SE = ‘n.a.’ indicates data available from only one tank of fish.

ND = no reportable data.

respectively) from diet even under long-term storage, and that 4500 p.p.m. CHL did not significantly alter the recovery (96.4 and 87.6%, respectively) of DBP doses of 28.4 and 225 p.p.m. (t-test: P > 0.1, both DBP doses).

Statistical methods

Mortality, apparent tumor multiplicity, tumor phenotype spectrum, adduct profile and diets were evaluated using single factor ANOVA and two-sample t-tests assuming unequal variances. Two-tailed P-values are stated. Liver somatic indices of the Sigma CHL-treated animals were evaluated using linear (ANOVA- and ANCOVA-type) models. Liver somatic indices of the trout treated with the powdered Derifil or placebo tablets were evaluated using t-tests using the pooled error mean square from the entire experiment.

Total adducts were initially modeled as a common background plus additional adducts proportional in DBP dose (K DBP × DBP) with the slope (K) allowed to vary for each level of CHL. Because variation increased with the response, quasi-likelihood was used with the variance proportional to the mean (multiple of Poisson distributed) or likelihood was used with the variance proportional to the square of the mean (gamma distributed). For stomach adduct data (with a smaller range of response), the model fit well using multiple of Poisson. For liver data variation was best modeled using quasi-likelihood and the residual variation was used to estimate a treatment-combination level dispersion parameter (degrees of freedom equal to the number of treatment combinations minus the total number of parameters in the model). In this context lack-of-fit could be accounted for by adjusting for any treatment effects (saturated model), covariates were added for linear and quadratic effects in day of sampling and in body weight.

When there was evidence of lack-of-fit to the parallel straight-line model for logit of tumor incidence as a function of CHL treatment and log of DBP dose (liver tumor data), more complex models were examined. When lack-of-fit was treated as random variation at the treatment-combination level (see Discussion), the additional random variation beyond the replicate tank variation was incorporated as follows: The data pooled over replicate tanks (one observation for each treatment combination) was modeled by quasi-likelihood and the residual variation was used to estimate a treatment-combination level dispersion parameter (degrees of freedom equal to the number of treatment combinations minus the total number of parameters in the model). In this context lack-of-fit could be assessed by adding additional parameters (e.g. interactions, quadratic terms). For the pooled data there was little evidence that mean body weight affected tumor incidence (P > 0.17).

For simple comparisons of tumor incidence for sparse data (e.g. swim bladder), Fisher’s Exact Test [Freq procedure in SAS (2003)] was used to: (1) confirm that there was no evidence of over-dispersion between replicate tanks and (2) compare treatments after pooling across replicate tanks.

For total adduct and tumor data, analyses were done in SAS (2003) using the Genmod, Glimmix and Nlmixed procedures (53).
sampling was observed (data not shown). However, with the exception of four tanks, the mean for each tank was within ± 2 SD from the overall mean, and there was no evidence for increased body weight influencing final liver tumor incidence \((P > 0.17)\). Liver somatic indices displayed a small decrease with time \((P < 0.01)\), and a significant linear decrease was found with increasing CHL concentration at the lowest dose of carcinogen, however, no significant overall CHL or DBP treatment-related trends were discernable. A small, unexplained elevated mortality, consistent across all the DBP doses, was noted at the 4500 p.p.m. CHL dose \((P = 0.01)\), however, there was no difference in the mortality rates among the other three higher or lower CHL doses and negative controls \((P = 0.2)\). There was no difference in mortality among the DBP doses and negative controls \((P = 0.2)\).

**DBP-initiated tumor formation and its modulation by CHL**

Administration of DBP in the diet of rainbow trout for 4 weeks resulted in a dose-dependent formation of tumors of the liver, stomach and swim bladder observed 10 months after DBP treatment ceased (Table I). Tumor incidence was greatest in liver at each DBP dose, followed by the stomach and then swim bladder. Addition of CHL to the diets generally resulted in a dose-dependent reduction in tumor formation across all doses of DBP. The most evident exception was seen in liver at 225 p.p.m. DBP, where CHL at \(\geq 4500\) p.p.m. was required to achieve observable reduction in incidence. Visual inspection of the unmodeled stomach (Figure 2A, inset) and liver (Figure 2B, main panel) data, plotted on a logit incidence versus log [DBP] scale, show a strong tendency of the DBP dose–tumor response curves to move to the right (toward higher log [DBP]) with increasing CHL concentration. That is, each CHL dose examined shifted the control DBP-only tumor response curve such that higher DBP doses were required to achieve any particular tumor response. These unmodeled results provide unambiguous evidence that CHL was an effective blocking agent when co-fed with DBP, and that cancer chemoprevention was achieved in both major organs at two or more CHL doses over the entire range of DBP doses administered.

Consistent with the findings of Reddy, et al. (37), the liver tumors arising as a result of initiation with DBP were primarily hepatocellular carcinomas (HCC) and adenomas (HCA), with relatively few mixed hepatocellular/cholangiocellular carcinomas (MC) or adenomas (MA) (Table I). This spectrum of trout liver tumor phenotypes is consistent with that reported previously (19,54) though the distribution of phenotypes can vary amongst carcinogens. In treatment groups where \(>20\) liver tumors were available for examination, there was no DBP dose-dependent change in the relative percentage of HCC, HCA or MC, the three most abundant phenotypes \((P \geq 0.1, \text{all three phenotypes})\). The phenotypic response in CHL co-treated groups was identical to that seen in response to DBP alone, regardless of the CHL concentration \((P \geq 0.1 \text{ for HCC, HCA and MC})\). All tumors of the stomach and swim bladder were papillary adenomas, irrespective of DBP or CHL dosage.

**Modeling of tumor responses**

One aim of the study design was to determine if the magnitude of CHL protection remained constant over all the DBP dose ranges. That is, we wanted to determine for each target organ if there is any basis for extrapolating the degree of CHL protection at high-carcinogen doses and incidences down to lower doses and tumor incidences perhaps more representative of human cancer risks. Use of a dose–dose matrix design permits the magnitude of cancer chemoprevention protection for any of several anti-carcinogen doses to be assessed by a modeling of the effect of each dose on the shape and position of the entire carcinogen dose–response curve (19,49). As we have previously shown, the condition of
equal magnitude of CHL chemoprevention over all the DBP doses would be met for any CHL dose at which the logit incidence versus log [DBP] dose–response curves with and without CHL are the same shape but offset horizontally (19,49). A related aim of the study design was to generate estimates for CHL dose–response potency to inhibit tumor formation at any or all the DBP doses. For this purpose the dose of carcinogen required to produce ρ% tumor incidence is defined as the TDp value. For instance, at ρ = 50%, the response curve for each dose of CHL will provide a TD50 estimate which can be used to calculate the magnitude of tumor inhibition for that dose of CHL using the following: percent inhibition = 100 (1 – (TD50CHL/TD50CHLX)) where CHL0 refers to the treatment with no CHL and CHLX refers to the treatment at any CHL dose X (19,49). We note that, when the dose–response curves are linear and parallel, the percent inhibition calculated from any tumor incidence within the linear range will be equivalent. In the case where the logit incidence versus log [DBP] dose–response curves with and without CHL are not linear, but differ only by being offset horizontally from each other, then there is still a constant ‘relative potency’ (55) of that dose of CHL over the range of DBP doses studied, and percent inhibition at that CHL dose can be calculated using the TD50 approach. If neither of these conditions applies, chemopreventive efficacy will have been demonstrated to vary with carcinogen dose as well as CHL dose, and calculations of % inhibition will produce no common, extrapolable value.

Stomach and swim bladder tumor response. The swim bladder data were sufficient to demonstrate strong CHL inhibition of DBP tumorigenicity in this minor target organ (Table I), however the very low tumor response in this organ provided incomplete dose–response curves that could not be further modeled. The stomach tumor response to various DBP doses was sufficient (Table I) to provide a full set of five unmodeled dose–response curves, although the low response resulted in one curve being represented by only two above-zero data points. The resulting curves (Figure 2A, inset) suggest a trend to the right (increasing DBP dose) with increasing dosage of CHL in this target organ. This suggestion was supported by successfully modeling the stomach tumor data by logistic regression as a series of linear and parallel dose–response curves (lack-of-fit P > 0.5), with each increase in CHL providing successive offsets toward higher TD values (Figure 2A, main panel). This finding supports the conclusion that the magnitude of tumor inhibition increased with CHL dose, and was independent of DBP dose at each of the four CHL doses studied. That is, the magnitude of CHL protection (% inhibition) measured at high-carcinogen dose applied equally at low dose within the DBP range studied, at every CHL dose examined.

Successful fit to the parallel-offset model also allowed quantification of CHL dose–response potency in this organ by calculating percent inhibition of tumor formation as above, using TDP values within the linear range of the dose–response curves. This calculation yields a dose–response potency for CHL % inhibition (± SE%) of 30 (± 9), 49 (± 7), 62 (± 5) and 69 (± 4) for CHL doses of 1500, 3000, 4500 and 6000 p.p.m., respectively (see Figure 3).

Liver tumor response. Prior to modeling the liver incidence data we note an aspect of the data that we have repeatedly observed in other studies: the dose–response in trout liver to DBP alone reached a plateau near 60% incidence at ~80 p.p.m. DBP on the log/logit scale, such that increasing DBP exposure to such higher doses as 225 p.p.m. provided negligible additional tumor response in this organ (Figure 2B, main panel). Since this data point (225 p.p.m. DBP, 0 CHL) is confirmed a priori not to lie within a linear dose–response range, it was excluded from modeling investigations testing logit linearity in response. The remaining 18 DBP–CHL treatment combinations were modeled using logistic regression with over-dispersion estimated from the replicate tank variation (quasi-likelihood because variation was ~21% larger than expected under the binomial assumption). A logit linear in log [DBP] dose model with common slope (parallel) and separate intercepts for each of the five CHL doses (to allow horizontal offset) was examined first. Although the parallel-line model (six parameters) can explain much of the pattern in the observed incidences, the remaining variation in the incidences about the model was too large to be explained by the replicate tank variation (strong evidence of lack-of-fit (LOF), 12 df, P < 0.0065 quasi-likelihood F-test). No simple model could explain the lack-of-fit. (e.g. allow separate slopes adding four parameters, or include a common quadratic curvature adding one parameter). A complex model requiring 15 parameters to fit 18 treatment combinations was required before LOF based on replicate tank variation became non-significant (separate linear and separate quadratic parameters for each curve, LOF 3 df, P = 0.15). Therefore, standard quasi-likelihood logistic regression modeling of these liver incidence data does not support the condition of equal magnitude of CHL chemoprevention in this organ over all the DBP doses, even though the unmodeled data (Table I and Figure 2B, main panel) provide clear evidence of CHL chemoprevention that is dose-responsive and observable at all the DBP doses examined. A third model allowing for additional random variation in this particular study at the level of the 18 DBP–CHL treatment combinations being modeled (above the replicate tank level) rather than true, systematic lack-of-fit in the underlying
dose–response relationships in the liver data set is presented in the Discussion section.

**CHL effects on apparent liver tumor multiplicity**

Because of the magnitude of the study we were unable to conduct exhaustive pathology on every lesion within livers bearing multiple grossly observable lesions. It was feasible, however, to examine the gross lesion data for possible effects of DBP and CHL dose on what we term apparent multiplicity. Reminiscent of the incidence data, apparent liver tumor multiplicity was not a simple linear or monotonic function of DBP dose, but instead exhibited a maximum response near 80 p.p.m. DBP (Figure 4). Increasing CHL co-treatment doses had the effect of shifting the observable portions of this curve progressively toward higher DBP dose. The net result is that all four CHL treatments were seen to reduce apparent multiplicity at DBP doses of 80 p.p.m. and lower, compared with DBP alone \( (P<0.05) \), whereas at 225 p.p.m. DBP the three lowest CHL doses enhanced apparent tumor multiplicity compared with DBP alone \( (P<0.05) \).

**DBP–DNA adduct formation, and inhibition by CHL**

Structural identification of each and every DBP–DNA adduct in liver and stomach was beyond the scope of this study. We were, however, able to use a post-labeling/HPLC protocol to resolve and quantify major and minor adduct peaks and thus examine the effects of DBP and/or CHL dose on adduct profile. This information can provide some insight into the mechanisms involved in adduct formation and its inhibition by CHL in this species. The profiles of DBP–DNA adducts recovered from trout liver and stomach (Figure 5) were virtually identical to those reported in our earlier study using an identical \(^{33}\)P-postlabeling protocol (56). The liver DBP–DNA adduct profile (Figure 5A) consists primarily of polar adduct peaks eluting within the 10–20 min range and several smaller non-polar peaks eluting in the 35–65 min range. Increasing concentrations of CHL at each dose of DBP resulted in a relatively uniform decrease in the adduct profile. This is illustrated in Figure 5B, which shows the magnitude and uniform nature of the decrease in DBP adduct profile at 1500 p.p.m. CHL. There was no evidence of a dose effect by either DBP \( (P>0.15) \) or CHL \( (P>0.2) \) on the adduct formation.

**Fig. 4.** CHL effects on apparent tumor multiplicity in liver. Trout were examined for tumor formation 11 months after initiation. Apparent multiplicity is used to denote the inclusion of some gross as well as histologically confirmed lesions among individuals with more than one lesion. Symbols represent the same CHL treatments as shown in Figure 2. Incremental doses of CHL are represented by alternating solid and dashed lines. The short and long dashed lines represent 1500 and 4500 p.p.m. CHL, respectively. Error bars represent standard deviation.

**Fig. 5.** Chromatograms of in vivo DBP–DNA adducts from rainbow trout after \(^{33}\)P post-labeling and HPLC analysis. Liver adduct profiles were generated by 4-week dietary exposure to either 225 p.p.m. DBP alone (A) or 225 p.p.m. DBP and 1500 p.p.m. CHL (B). Stomach adducts resulting from 4-week dietary exposure to either 225 p.p.m. DBP-only (C), or 225 p.p.m. DBP and 1500 p.p.m. CHL (D) are shown.
profiles, only in total adduct amounts. In the stomach, the majority (generally >70%) of DBP–DNA adducts eluted in the non-polar 35–65 min range, with very few adducts in the polar 10–20 min range (Figure 5C). As in the liver, addition of CHL to the diets resulted in a largely uniform decrease in peak height across the stomach adduct profile (Figure 5D). A minor exception is noted in that the addition of the lowest dose of CHL resulted in a small, but statistically significant ($P = 0.003$) increase in polar DNA adducts in the stomach, despite a decrease in total adducts recovered (Table II). This small elevation in percentage of polar adducts and corresponding decrease in non-polar adducts ($P \leq 0.02$) was also seen at higher CHL concentrations (for polar adducts, $P \leq 0.01$ at 4500 and 6000 p.p.m. CHL), however, it was considerably smaller in magnitude relative to overall reduction in adduct formation. In all, these data do not suggest significant DBP dose-dependent changes in metabolic pathways leading to DNA adduct formation in either target organ, or CHL dose-dependent modulation among those pathways, over the range of DBP and CHL dosages in this study. Therefore, for subsequent analyses all non-background peaks beyond flow-through were included in the calculation of total adducts for each treatment replicate.

Using this procedure, 4 weeks of dietary DBP treatment was determined to produce a dose-dependent formation of total DBP–DNA adducts in trout liver and stomach (Table II). Figure 6 models the DBP dose–response data set for adduct formation, and the effect of CHL co-treatment in reducing adduct formation, for each organ. In stomach, adducts in the DBP-only groups accumulated linearly with DBP dose (Figure 6A). In the liver, adducts accumulated in a manner fitting an upwardly curving model,

$$\text{adducts} = 1.36 + (\text{slope}_{\text{CHL}}) ([\text{DBP}]^{0.22})$$

(Figure 6B). Addition of 1500 or 3000 p.p.m. CHL produced substantial reductions in the slope of the positive control DBP dose–DNA adduct response line in liver as well as stomach, whereas 4500 and 6000 p.p.m. CHL provided somewhat less additional protection in either organ. The CHL-mediated percentage inhibition of adduct formation can be determined by evaluating the change in slope of the modeled data using

$$\%\text{inhibition} = 100 \times (1 - ([\text{slope}_{\text{CHLX}}]/[\text{slope}_{\text{CHL0}}])),$$

where, CHLX = CHL at dose X and CHL0 = control. Using this formula, the reduction (± SE) in liver adducts at CHL doses of 1500, 3000, 4500 or 6000 p.p.m. were 49 (± 9), 69 (± 6), 81 (± 3) and 83% (± 3), respectively. In the stomach, the adduct response was inhibited by 47 (± 8), 61 (± 7), 72 (± 4) and 75% (± 4) at the same respective doses of CHL, results similar to inhibition of liver adduction.

**DBP–DNA adducts as predictive biomarkers of chemoprevention**

We next asked if the CHL-mediated reduction in DBP–DNA adduct formation may provide an appropriate early biomarker to predict CHL effects on final tumor outcome. There are several approaches that can be used for this assessment (49,57). The simple, unmodeled molecular dosimetry relationship between log [DNA adducts] and logit incidence is shown separately in both stomach (Figure 7A) and liver (Figure 7B) for each combination of CHL and DBP dose (the combined data from both organs is shown in the inset of Figure 7B). This analysis has the advantage of making no assumptions with regard to the linear nature of the tumor or adduct dose–response curves. Several conclusions are evident from the figure. In liver, increased DBP–DNA adduction above ~4000 nmol adducts/mol DNA produced no further increase in tumor incidence above 60%. Conversely, at high-DBP dose (225 p.p.m.), CHL co-treatment reduced tumor incidence below the 60% plateau only at those (high) CHL doses capable of reducing DNA adducts to <4000 nmol adducts/mol DNA; although 1500 and 3000 p.p.m. CHL reduced adducts by 40 and 80%, respectively (Table II), this was not accompanied by a reduced tumor outcome. Below the 4000 nmol adduct/mol DNA level, the liver and stomach data appear consistent with a simple linear relationship...
between adducts and tumor incidence (Figure 7B, inset, $r^2 = 0.87$). However, direct 1:1 proportionality would be demonstrated only by a regression line of slope 1.0 on the log/log scale. When this is done, the liver data ($slope = 1.35, r^2 = 0.87$) and, to a lesser extent, the combined data (liver + stomach; $slope = 1.70, r^2 = 0.86$) approach direct proportionality, but the stomach data alone do not ($slope = 2.03, r^2 = 0.90$). That is, tumor incidence in stomach changes in proportion to the square of adducts but, within this relationship, CHL-mediated alterations in adducts still correlate with final tumor outcome.

An alternative description of the relationship between CHL dose, adduct formation and tumor response is provided by the fractional inhibition analysis depicted in Figure 7C. By this analysis data points lying on the unity slope diagonal represent CHL doses at which the extent of inhibition of DNA adducts (represented by $DBP_{CHL}/DBP_0$) precisely predicts the extent of reduction in eventual tumor incidence (represented by $TD_{50\text{control}}/TD_{50\text{CHL}}$). Data points lying significantly above the diagonal represent CHL treatments at which adduct reduction significantly overestimates eventual tumor reduction, whereas points below the line indicate adduct reduction that underestimates inhibition of eventual tumor response. As shown in Figure 7C, CHL effects on DBP adducts in stomach were somewhat over-predictive biomarkers, that is, adduct reduction systematically overestimated tumor reduction by ~35% over all CHL treatment doses in this organ. The basis for this is unclear at present. This analysis for liver could be carried out only through an alternative modeling to provide $TD_{50}$ estimates (see Discussion). In this organ, CHL-mediated reductions in total adducts were precisely predictive early biomarkers of eventual CHL reduction in liver tumor response at the three higher CHL doses, and a conservative biomarker that underestimated tumor inhibition at the lowest CHL dose tested.

Response to CHL and placebo clinical formulations

Along with the Sigma CHL preparation, we had the opportunity to include in our evaluation the CHL tablet (Derifil) and placebo from the same lot used in the biomarker clinical intervention trial in an AFB$_1$-endemic area of China (26). Both Derifil CHL and placebo tablets were powdered, mixed into the diets, and evaluated for effectiveness at a single 80 p.p.m. DBP dosage dose. The level of Derifil CHL was chosen to approximate the total Cu CHLs in the 4500 p.p.m. Sigma CHL diets. Neither the Derifil nor the placebo treatment was associated with a change in liver somatic index ($P > 0.5$ and $>0.3$, respectively). As shown in Table I and Figure 2A and B, inclusion of the powdered Derifil tablets substantially reduced liver and stomach tumor incidences. Fish receiving Derifil and 80 p.p.m. DBP had significantly fewer adducts ($P < 0.002$) than did fish receiving 4500 p.p.m. CHL and 80 p.p.m. DBP. The placebo formulation

**Fig. 7.** Quantitative relationships between initial DNA adduct formation and final tumor incidence. Panels A and B depict the unmodeled molecular dosimetry of log [DNA adducts] versus log [final tumor incidence] in stomach (open symbols) and liver (filled symbols), respectively. The combined stomach and liver data are shown in the inset of panel 6B. Diets contained 10.1, 28.4, 80, 225, or 371.5 p.p.m. DBP and 0 (open circles), 1500 (open upward triangles), 3000 (open squares), 4500 (open diamonds), or 6000 (open downward triangle) p.p.m. CHL. Additional treatments consisted of 80 p.p.m. DBP plus either Derifil (filled hexagon) or placebo tablets (cross inside circle). The regression line shown in the inset combines the liver and stomach data to explore the hypothesis that alteration in adducts accounts for altered tumor response in both organs ($r^2 = 0.86$, slope = 1.70, see text). Treated individually, regressions for liver and stomach also reveal strong correlations but the two lines have different slopes (liver, $r^2 = 0.87$, slope = 1.35; stomach $r^2 = 0.90$, slope = 2.03. Log-log scale, data not shown). Data points and error bars represent means and standard errors, respectively, from replicate tanks at each dose. Errors were calculated prior to log and logit transformations. No stomach tumors were present and no data was plotted for DBP and CHL dose combinations 10.1 and 0, 10.1 and 3000, and 28.4 and 3000 p.p.m., respectively, however, all data points were used for modeling. Because of the logit-scale distortion at low tumor incidences, the lower error bar for the remaining two 3000 p.p.m. CHL data points have been omitted from the displayed stomach data. Panel C shows the relationship between CHL fractional inhibition of DBP–DNA adduction and fractional inhibition of tumorigenesis for four doses of CHL in liver and stomach and standard error (see text for derivation of values). Liver tumor inhibition values were determined as described in the Discussion section.
unexpectedly showed a moderate, but significantly lower liver tumor response (38 versus 57% incidence, Table I), when directly compared with the 80 p.p.m. DBP-only control ($P < 0.001$). This apparent inhibition by the placebo was less than that seen with the lowest dose of Sigma CHL (1500 p.p.m.) in this study. This apparent effect was not seen in stomach, where there was no evidence of a difference in tumor response between those animals fed 80 p.p.m. DBP plus placebo and the 80 p.p.m. DBP-only control ($P > 0.5$), nor was there evidence of a difference in adduct levels between those fish receiving Derifil or 4500 p.p.m. CHL ($P > 0.3$). In the swim bladder, no tumors were produced in the Derifil treated animals and there was no evidence of a difference in the swim bladder tumor incidence between CHL $= 0$ and placebo ($P > 0.05$ Fisher’s exact test).

As with the Sigma CHL, there was no significant effect on mean body weight or tumor spectrum with either the Derifil or placebo preparations ($P > 0.1$ for all comparisons). Table II shows the quantity of DBP–DNA adducts detected in the liver and stomach following co-treatment with the Derifil CHL and also for the placebo formulation in the stomach. Derifil CHL provided an 86% reduction of DBP–DNA adducts in liver ($P < 0.01$), compared with the 70 and 76% reductions shown by 4500 and 6000 p.p.m. Sigma CHL. The evident 48% reduction ($P = 0.07$) in mean stomach adduct formation following Derifil CHL treatment was similar to that seen with the 3000 p.p.m. CHL treatment. In this organ, the quantity of adducts recovered following placebo treatment was not different from the DBP-only control ($P = 0.9$). The Derifil and placebo responses were also included in the molecular dosimetry figures for both the liver and stomach (Figure 7A and B). Here, both the Derifil CHL and stomach placebo data appear to mingle with the other data from this study, indicating that DBP–DNA adduction following treatment with these preparations was also approximately predictive of tumor outcome. There was evidence of a Derifil-associated reduction in tumor multiplicity (2.43 ± 1.36), however, the placebo treatment (3.83 ± 0.17) was also associated with a reduction in gross tumor multiplicity relative to the 0 p.p.m. CHL control (Figure 4) such that the responses to the Derifil and placebo formulations were statistically indistinguishable from each other ($P = 0.3$).

Discussion

**CHL dose-responsive potency for tumor inhibition**

The present chemoprevention study employed over 12 000 animals in a dose–dose matrix design to evaluate the protective effects of dietary CHL on DBP multi-organ DNA damage and tumorigenicity. Qualitatively, CHL co-exposure was seen to provide dose-dependent reduction in final tumor incidence in three target organs (liver, stomach and swim bladder), and over a 20-fold range of DBP exposures (Table I). In quantitative terms, dietary CHL reduced stomach tumor response to DBP from 30 up to 68% based on successful modeling of the entire stomach data set as a series of parallel dose–response curves displaced toward higher TD$_{50}$ with increasing CHL (Figure 2A).

Although CHL appeared to exhibit similar potency for inhibition of liver tumors, the parallel-offset model failed primarily due to an observed sub-linearity response to the lowest DBP dose in the DBP-only treatment groups in this particular study. However, a concurrent very large study with 42 000 trout established a linear logit in log dose–response over the 10–80 p.p.m. DBP dose range (Bailey et al., manuscript in preparation), suggesting that the complex lack-of-fit only represented additional random variation in this particular study at the level of the 18 DBP–CHL treatment combinations being modeled (above the replicate tank level) rather than true, systematic lack-of-fit in the underlying dose–response relationships. Therefore, a final parallel-offset dose–response model for liver was examined by treating lack-of-fit as random variation at the treatment-combination level, and the additional random variation beyond the replicate tank variation was incorporated into the parallel straight-line model in a simple manner. The data pooled over replicate tanks (one observation for each treatment combination) was modeled by quasi-likelihood and the residual variation was used to estimate a treatment-combination level over-dispersion parameter (degrees of freedom (df) equal to the number of treatment combinations minus the total number of parameters in the model). Figure 2B (inset) shows the fit of the parallel dose–response model by this approach, and Figure 3B shows the results for liver when that fit is used to estimate average relative potencies for each positive dose of CHL while incorporating the likely additional random variation at the treatment-combination level. By this approach the dose–response potency for CHL % inhibition ($\pm$ SE) was 62 ($\pm$ 6), 67 ($\pm$ 5), 77 ($\pm$ 3) and 82% ($\pm$ 3) at 1500, 3000, 4500 and 6000 p.p.m. CHL inhibition, respectively (Figure 3B), and appears to be somewhat greater in liver than stomach, especially at lower CHL dose. Such a difference in the two organs would be contrary to the hypothesis that CHL inhibits primarily through reduction of carcinogen uptake.

**Unmodeled comparisons at high-DBP dose**

These models of CHL effects on the positions of entire DBP dose–response curves afforded the conclusion that even modest doses of 1500–3000 p.p.m. CHL during the period of carcinogen exposure provided strong protection against DBP tumorigenicity in liver as well as stomach. However, different quantitative or qualitative conclusions can be reached if CHL inhibition is considered at selected single DBP doses, as are typically employed in rodent chemoprevention study designs. For instance, using simple tumor incidence ratios derived from Table I, one can calculate that CHL doses of 1500, 3000, 4500 and 6000 p.p.m. provided 55, 63, 75 and 78% ‘inhibition’, respectively, among groups receiving 80 p.p.m. DBP. Therefore, a study carried out only at 80 p.p.m. DBP would lead to the same overall conclusion as the present study, that CHL is a highly effective blocking agent, even at doses down to 1500 p.p.m. However, among groups receiving the higher 225 p.p.m. DBP exposure, these same doses of CHL provided only 6, 6, 32 and 55% ‘inhibition’ by this kind of calculation. Hence a study using only 225 p.p.m. DBP (in an attempt, for instance, to maximize tumor response in the carcinogen-only group) would lead to the erroneous conclusion that CHL is not a very potent blocking agent, requiring doses of 4500 p.p.m. or greater to show significant protection.

This result demonstrates that the conclusions reached regarding chemopreventive efficacy can be highly dependent on carcinogen dose chosen. Such an anomaly occurs in
the present study because hepatic tumor incidence was not proportional to DBP at high-dose, i.e. was non-linear. A separate 42 000-trout study has confirmed this non-linearity, and suggests a significant decrease in liver cell proliferation at high-DBP dose as a possible basis for saturation of hepatic response in this model (Bailey, et al., manuscript in preparation). Non-linearity in DBP dose–response for liver tumor multiplicity is even more problematic. Here, CHL was clearly protective at lower DBP doses, but because of the inverted u-shape of the DBP dose–response curve, three of the four CHL doses tested actually enhanced multiplicity at 225 p.p.m. DBP. A recently completed 12 000 animal study again confirms the saturation of liver tumor incidence (Figure 2B) and the decline from peak tumor multiplicity (Figure 4) as dietary DBP dosage exceeds 160 p.p.m. in this model (Bailey, et al., manuscript in preparation). The basis for this is presently unclear, but it is not due to enhanced DNA repair or metabolic changes leading to a decreased rate of DNA adduct accumulation (Figure 6B) nor to any changes in the relative quantities of each DNA adduct at high versus low DBP dose (Figure 5; data not shown).

Since humans are unlikely to be exposed to a chemical carcinogen inducing 60% tumor incidence or multiplicities approaching 5.0, we believe that the chemoprevention data at lower DBP doses, incidences and multiplicities are more indicative of CHL utility in human chemoprevention, and they demonstrate CHL inhibition rather than enhancement of tumor response.

**DNA adduct reduction as a quantitative biomarker for tumor inhibition**

Dietary CHL provided dose-responsive decreases in DNA adduct formation in liver and stomach at all the DBP doses examined. The usefulness of these early adduct data as predictive biomarkers of CHL reduction in eventual tumor outcome can be evaluated by several means.

In our previous analysis of CHL chemoprevention against AFB1 hepatocarcinogenesis using the rainbow trout model (19), fractional reduction analysis showed that the extent of tumor protection (based on TD50 ratios) was predicted by changes in AFB1–DNA adduction (based on DNA binding index or DBI ratios) at lower CHL doses, but that tumor protection at higher CHL was significantly greater than would have been predicted from DNA adduct protection. This was interpreted to indicate that additional chemoprotection mechanism(s) occurred at higher doses of CHL, reflecting dose-dependent alterations in the initiation process beyond those correlated with AFB1–DNA damage alone.

The present study provides no evidence that this is a general feature of CHL chemoprevention at higher dose. In Figure 7, which describes the molecular dosimetry relationships at all the CHL doses, the collective data (panels A and B) appear to describe a common logit (incidence)/log (adduct) molecular dose–response line in stomach and in liver (Figure 7B, inset). The most evident outliers in the data set were seen for liver at 225 p.p.m. DBP, 0 and 1500 p.p.m. CHL, where the DBP dose-related increases in DBP–DNA adduction were not accompanied by a proportional increase in eventual tumor response. This deviation at high-DBP dose has been previously observed in this model, and may reflect the saturation of liver tumor response at ~60%, perhaps due to reduced cell proliferation in liver at high-DBP doses in trout liver (Bailey, et al., manuscript in preparation).

Interestingly, the DBP adduct/incidence data point in stomach at 225 p.p.m. DBP (Figure 7A) did not show any apparent plateau effect in either study, nor have we seen a high-DBP dose effect on proliferation in this organ in trout. With the exception of the 1500 p.p.m. CHL dose, there is no indication that the CHL doses in this study resulted in an adduct/incidence data set lying consistently to the right (or the left) of the general trend in liver (Figure 7B and C), as was previously observed using AFB1 as carcinogen (19,49). The relationship in stomach between inhibition of DBP–DNA adduction and inhibition of tumorigenesis (Figure 7A) suggests that reductions in DNA adduct formation systemically overestimated CHL-mediated protection from tumor formation by ~35% over all the CHL doses evaluated. The reason for this is unclear at present, however, this observation suggests that DNA adducts in stomach are optimistic biomarkers and will consistently underestimate subsequent tumor response in this organ. The low costs of the trout model permit a sufficiently large number of animals and treatment groups to allow these conclusions to emerge; evidence from any single-dose comparison, for example examination of CHL dose effects at 80 p.p.m. DBP dose, yields only a simple correlative relation between CHL-mediated DBP–DNA adduct reduction and eventual tumor response. The empirical data from this study do not directly address the reasons for every departure from a 1:1 relationship between adduct formation and final tumor response, but support the growing body of evidence that additional mechanisms are occurring that are not the direct consequences of initial levels of carcinogen–DNA damage. In a separate study we report the many initial and persistent alterations in gene expression that occur in trout liver and liver tumors following carcinogen exposure, and the similarity in these alterations to those in rodent and human liver cancers (58).

**Clinical application for CHL in cancer prevention**

Also evaluated in this study were oral formulations containing 100 mg CHL in a tablet sold commercially as Derifil as well as a visually identical, green-coated placebo tablet, which contained the same excipient but no CHL. These tablets were from the same lot as those used in a double-blinded, placebo-controlled intervention clinical trial in which oral administration of the CHL tablets was associated with a 55% reduction in the amount of urinary AFB1–N7-guanyl DNA repair product (26) that forms in response to AFB1 exposure (59,60). In rainbow trout initiated with dietary DBP, dietary co-treatment with this tablet formulation resulted in significant reduction in DNA adduct formation and final tumor incidence in both liver and stomach. Additionally, the protection afforded by the Derifil formulation was apparently greater than that seen in the 4500 p.p.m. Sigma CHL preparation which was calculated to contain the same concentration of chlorins. An explanation for this may lie in the differing compositions of reagents called ‘chlorophyllin’ (CHL). The fact that CHL preparations consist of mixtures of chlorins with potentially different biological activities has been recognized and discussed (61,62). Interestingly, the placebo formulation was also found to provide some protection against DBP–DNA adduction and tumor formation in liver. It is possible, for instance, that this might reflect some true inhibitory activity of the green dye used to coat the placebo tablets. One implication is that
the efficacy of CHL for biomarker reduction in the human intervention trial (26) might have been even greater than that reported, had a biologically neutral but visually efficacious placebo been available. The potential for chemoprotection by the green coating dye used in the placebo formulation has not been investigated, nor has the present study been repeated in the trout or other animal models.

No quantitative evaluation of CHL composition or content in target tissues in rainbow trout was undertaken, however, the visual observation of green livers during sampling for DNA adducts in those animals actively receiving both Sigma and Derifil CHL is evidence of systemic absorption of at least some components of CHL in both preparations. Elsewhere, an in vitro analysis of CHL stability indicated chlorin e6 was substantially more stable than chlorin e4 under conditions of simulated digestion (63). This observation offers an explanation for detection of chlorin e4 in human serum to the exclusion of chlorin e6 (45) and suggests a reason why the Derifil preparation was apparently more potent than the Sigma preparation in the present study. The presence of a food matrix reportedly reduced the digestive degradation of chlorin e6 substantially (63) leaving open the possibility of a protective role for unabsorbed chlorin e6 in the digestive tract following dietary co-treatment as evaluated here in the rainbow trout model.

In summary, the present study used an initial 12350 animals to successfully explore the complex interrelationships between carcinogen dose applied, anti-carcinogen dose applied, target organ DNA adduct biomarkers (effective dose received), and eventual tumor outcome. By molecular dose analysis, CHL-mediated alterations in DBP–DNA adduct levels were generally predictive of eventual reduction in hepatic tumor incidence and multiplicity, but only below 80 ppm DBP. At higher carcinogen dose, however, lower doses of CHL failed to reduce hepatic tumor incidence, all but the highest CHL dose actually elevated tumor multiplicity, and DNA adducts failed to be predictive biomarkers of eventual tumor outcome. These results demonstrate the importance of establishing endpoint dose–response proportionality in selecting carcinogen doses for chemoprevention studies. High doses lying beyond the range of proportional response were shown herein to give results that either seriously underestimated anti-carcinogen protective potency (liver incidence) or, worse yet, failed to predict the direction of chemopreventive action (liver multiplicity) at carcinogen doses and tumor rates more pertinent to human intervention.

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Conflict of Interest Statement: None declared.

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