Predictive Value of Molecular Dosimetry: Individual versus Group Effects of Oltipraz on Aflatoxin-Albumin Adducts and Risk of Liver Cancer


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Abstract
Studies in animals and humans have established serum aflatoxin-albumin adducts as biomarkers of exposure to aflatoxin B₁ (AFB₁), a food-borne hepatocarcinogen. To assess the utility of measurements of aflatoxin-albumin adducts to predict risk of hepatocellular carcinoma (HCC), 123 male F344 rats were dosed with 20 μg of AFB₁ daily for 5 weeks after randomization into three groups: no intervention; delayed-transient (500 ppm of oltipraz, weeks 2 and 3 relative to AFB₁); or persistent (500 ppm oltipraz, weeks 1 to 5). Serial blood samples were collected from each animal at weekly intervals throughout aflatoxin B₁ exposure and assayed for levels of aflatoxin-albumin by radioimmune assay. Area under the curve (AUC) values for aflatoxin-albumin adducts decreased 20 and 39% in the delayed-transient and persistent oltipraz intervention groups, respectively, as compared to no intervention. Similarly, the total incidence of HCC dropped from 83 to 60% (P = 0.03) and 48% (P < 0.01) in these groups. Tumor multiplicity was also reduced in the two oltipraz intervention groups, whereas time to HCC was increased. Mononuclear cell leukemia, a common neoplasm in F344 rats, was seen in 39% of the control animals, whereas the two oltipraz interventions reduced incidence to 18% (P = 0.05) and 13% (P = 0.01), respectively. Overall, a significant association was seen between biomarker AUC and risk of HCC (P = 0.01). However, when the predictive value of aflatoxin-albumin adducts was assessed within treatment groups, there was no association between AUC and risk of HCC (P = 0.56). Thus, aflatoxin-albumin adducts can be useful for monitoring population-based changes induced by interventions, such as in chemoprevention trials, but have limited utility in identifying individuals destined to develop HCC. As a consequence, the use of this biomarker in quantitative risk assessment should be pursued cautiously.

Introduction
Chemical-specific markers have been developed for a number of environmental carcinogens for use as molecular dosimeters of individual exposures (1). These markers provide the prospect of contributing substantially to the specificity and sensitivity of epidemiological studies aimed at determining the role of environmental agents in the etiology of human cancers (2, 3). Some of these biomarkers may also assist the monitoring of populations for changes in cancer risk, as may be achieved through exposure avoidance and chemoprevention (4). Biomarkers of the biologically effective dose may be particularly useful in this context in that they, in theory, provide a mechanistic linkage between exposure and disease outcome. The biologically effective dose reflects the amount of carcinogen that has interacted with its critical macromolecular targets and has been measured as either carcinogen-DNA or carcinogen-protein adducts. However, despite their increasing prominence, there are few instances where the promise of these biomarkers has been fulfilled. This reflects both the actual infancy of their development as well as intrinsic limitations to their application in populations (5, 6). To date, no carcinogen-specific biomarker has undergone full validation to quantitatively define the extent of the relationships between external exposure, biomarker levels, and cancer outcome.

Studies with the aflatoxins have served as a guidepost in the development of such molecular approaches in the assessment of exposure status and risk of HCC, one of the most common cancers in Asia and sub-Saharan Africa. Over the past 30 years, there have been extensive efforts to investigate the association between aflatoxin exposure and HCC (7). These studies have been generally hindered by the lack of adequate dosimetry data on aflatoxin intake, excretion, and metabolism in humans, as well as by the general poor quality of worldwide cancer morbidity and mortality statistics. However, recent prospective studies using biomarkers of the biologically effective dose of aflatoxin have provided striking confirmation of the postulated associations derived from the earlier ecological and

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3 The abbreviations used are: HCC, hepatocellular carcinoma; CI, confidence interval; AFB₁, aflatoxin B₁; AUC, area under the curve; MCL, mononuclear cell leukemia.
observational studies. Using urinary aflatoxin biomarkers, Qian et al. (8) demonstrated a dramatic synergistic interaction between aflatoxin and infection with hepatitis B virus in the risk of HCC in residents of Shanghai, People's Republic of China. The presence of urinary aflatoxin biomarkers alone resulted in a significant 3.4-fold increase in risk (95% CI, 1.1–10.0). Unlike measurements of urinary excretion of aflatoxins, which measure very recent exposures to aflatoxins, the determination of serum levels of aflatoxin-albumin adducts appears to provide a more integrated assessment of aflatoxin exposures over a several-month period. A recent longitudinal study in 120 residents of Daxin Township in Qidong, People's Republic of China, demonstrated the consistent presence of aflatoxin-albumin adducts in most participants (9). A nested case-control study in a prospective cohort of individuals in Qidong who are seropositive for hepatitis B surface antigen has indicated that the relative risk for HCC among individuals also positive for aflatoxin-albumin adducts was 2.4 (95% CI, 1.2–4.7; Ref. 10). Similarly, in Taiwan, Wang et al. (11) observed an adjusted odds ratio of 2.8 (95% CI, 0.9–9.1) for detectable compared to nondetectable aflatoxin-albumin adducts in hepatitis B surface antigen-seropositive men.

As a component of the ongoing validation of aflatoxin biomarkers, the present study has sought to expand these important findings by determining the quantitative relationships between levels of aflatoxin-albumin adducts and individual risk for the development of HCC. This study has used a rodent model in which one major variable, carcinogen exposure, has been held constant and risk outcome has been attenuated through the use of persistent and delayed-transient interventions with the chemopreventive agent oltipraz. Serial measurements of aflatoxin-albumin adducts throughout the period of carcinogen exposure have allowed for an analysis of the longitudinal association between biomarker levels and individual development of liver cancer. Such studies, in turn, facilitate the objective evaluation of the use and limitations of molecular biomarkers to predict disease outcomes in individuals at high risk from exposure to environmental toxicants.

Materials and Methods

Chemicals. Oltipraz was obtained from the Chemoprevention Branch, National Cancer Institute, and was determined to be >99% pure by high-performance liquid chromatography (12). AFB₃, was obtained from Sigma Chemical Co. (St. Louis, MO) as was purified rat albumin and kits for albumin determination. All other chemicals and reagents were of the highest commercially obtainable quality.

Animals, Diets, and Treatments. Male F344 rats (100 g; Harlan, Indianapolis, IN) were housed under controlled conditions of temperature, humidity, and lighting. Food and water were available ad libitum. Purified diet of the AIN-76A formulation (Teklad, Madison, WI) without the recommended addition of 0.02% ethoxyquin was used, and fresh diet was provided to animals at least every other day. Oltipraz at a final concentration of 0.05% was mixed into the AIN-76A diet with a V-blender, and diet was stored at 4°C. Rats were acclimated to the AIN-76A diet for 1 week before beginning the experiment. At this time, 123 rats were randomized to three treatment groups: no intervention; delayed-transient intervention with oltipraz during weeks 9 and 10 of age; and persistent intervention with oltipraz during weeks 7–13 of age. All rats received 20 μg of AFB₁ in 100 μl of DMSO by gavage each morning during weeks 8–13. All animals were weighed weekly.

Analysis of Aflatoxin-Albumin Adducts in Serum. Approximately 100–150 μl of blood from each animal were collected from the tail vein in hematocrit tubes at weekly intervals beginning 1 week after the first dose of carcinogen and extending to 1 week after the last dose. Samples were collected 2 h after the daily administrations of AFB₁. The hematocrit tubes were subsequently centrifuged at 10,000 × g, and the resulting serum was decanted into Eppendorf tubes and stored at −70°C until assay. Serum samples were first concentrated by high speed centrifugation using Amicon Microcon-50 microcentrers. Briefly, 50 μl of rat serum were loaded onto a prewashed filter containing 300 μl of PBS and centrifuged at 8000 rpm for 15 min. Each serum sample was then washed with an additional 200 μl of PBS and recentrifuged. The concentrated sample was pipetted from the filter, and the volume was accurately measured. The amount of serum albumin in each sample was determined by a bromocresol purple dye binding method using rat albumin as standard (Sigma). Total protein content was determined by the method of Bradford (Bio-Rad Laboratories, Hercules, CA). Serum proteins were digested overnight at 37°C with nuclease-free Pronase (Calbiochem-Novabiochem Co., La Jolla, CA) in a ratio of 1:4 (w/w, enzyme:total protein) as described by Shebar el et al. (13). Two volumes of ice-cold acetone were added to the tubes followed by incubation at 4°C for 1 h to precipitate the proteins. Following centrifugation at 12,000 rpm, the supernatant was decanted into an Eppendorf tube and concentrated under N₂ to remove the acetone. Volumes were accurately adjusted with PBS to reflect a final protein concentration of 10 mg/ml. Samples were stored at −70°C prior to assay.

To minimize potential biases afforded by assay variability over the course of the analyses, longitudinal sets of serial samples from three animals in each of the treatment groups were assayed in each assay set. A radioimmun assay was used to quanitate aflatoxin-albumin adducts in duplicate rat serum digests based upon the method described by Wang et al. (9). Prior to assay, all samples were adjusted to contain a final concentration of 1 mg of protein/ml using digested protein prepared from a normal rat serum pool. Nonspecific inhibition in the assay was determined from this normal rat serum pool, and the average value was subtracted from all study samples. Standard curves for the radioimmu assays were determined using a nonlinear regression method described by Gange et al. (14).

Analysis of Cancers. All moribund rats were autopsied when clinical observations indicated that the rat would not survive. The criterion for autopsy was the inability of the rat to right itself or walk. This ataxia was usually accompanied by a significant (>15%) and rapid loss in body weight. All remaining rats were euthanized and autopsied 20 months after the first dose of AFB₁. All grossly abnormal tissues were taken for histopathological analysis. This included all spleens weighing >2 g (i.e., >0.5% body weight). Standardized sections of liver were cut from the two largest lobes from all livers that appeared normal; most normal-appearing spleens were also sampled. All tissues were fixed in formalin, processed by routine methods, and embedded into paraffin, sectioned, and stained with H&E. Hepatic foci, adenomas, and carcinomas were identified by two observers blinded to the identity of the treatments.

Statistical Analyses. The relationship of treatment group and outcome was quantified using both incidence and time-to-event ("survival analysis") measures. Separate analyses were conducted for the primary outcome of HCC by considering whether the animals had either any or multiple carcinomas separately. Incidence of all hepatic lesions (foci, adenomas, and carcinomas) as well as MCL in the three protocols were compared using Fisher’s exact test for independence (15). The relation-
ships between aflatoxin-albumin adduct levels and incidence was evaluated using standard logistic regression models. Adduct levels from each weekly serum collection and each summary measure (initial rate, peak, and AUC) were evaluated in separate regression models to determine their univariate association with cancer risk.

To assess the degree to which treatment modified the risk of HCC through the biomarker, the bivariate relationship of the treatments and biomarker levels in predicting disease risk was investigated. Specifically, the strength of association between treatment and outcome in logistic regression models with treatment as the only covariate and with both treatment and biomarker as covariates was examined. Notationally, the models were of the following forms:

$logit (Pr(HCC)) = \alpha + \beta_1 I(Delayed-Transient) + \beta_2 I(Persistent)$

$logit (Pr(HCC)) = \alpha + \beta_1 I(Delayed-Transient) + \beta_2 I(Persistent) + \gamma X$

where $I()$ is an indicator function for intervention group; $\beta_1$ and $\beta_2$ represent the univariate log-odds of HCC for the delayed-transient and persistent intervention groups relative to the control group; and $\beta_1$* and $\beta_2$* represent the log-odds after adjusting for the biomarker $X$. Thus, the reduction of the magnitude of coefficients $\beta_1$ and $\beta_2$ to $\beta_1$* and $\beta_2$*, respectively, reflects the degree to which the biomarker $X$ is a surrogate for the relationship of cancer risk and intervention. The two extremes that could occur would be: (a) given the level of biomarker, one does not need to know the intervention that was received (i.e., $\beta_1$ and $\beta_2$ significantly different from zero, but $\beta_1$* and $\beta_2$* are not); and (b) once intervention is known, the level of biomarker does not provide any additional information on risk of disease (i.e., $\gamma$ not significantly different from zero).

Separate survival analyses were conducted to investigate the time to the occurrence of either any or multiple carcinomas. The time from initial aflatoxin exposure to the presence of either of these events was considered censored for rats who were sacrificed either prior to the administrative end of the study (20 months from first exposure) that did not show the HCC outcome (i.e., any or multiple tumors) or who did not display ataxia or pronounced weight loss prior to the end of the study (regardless of subsequent carcinoma diagnosis). For analyses of multiple HCC lesions, animals showing single tumors were treated as right-censored (multiple tumors could occur after the single tumor event). Each animal with no carcinoma present at sacrifice was assumed to be at risk for the occurrence of carcinoma at any time past the censoring time. Because hepatic tumorigenesis is a relatively slow event, the assumption that HCC would occur immediately after the censoring time may not be appropriate. Thus, analyses using transformed censoring times were conducted; these times were computed by adding a conservative constant to the censoring time of 180 days for observations with no lesion or hepatic foci and 30 days for observations with hepatocellular adenoma. Using these outcome and censoring features, standard Kaplan-Meier methods (for any or multiple HCC events; Ref. 16) were used to graphically depict the time to HCC among the three groups. To evaluate the relationship between intervention groups and biomarker levels both separately and simultaneously on the time to HCC events, parametric log-normal regression models were fit for the time to HCC outcomes (16). These models have an advanced over standard Cox proportional hazards models in that they naturally parameterize the percentiles of the survival distribution, where the $p$th percentile is the time at which $p$ percentage develop HCC (hence 50th percentile = median). Additionally, it is easy to compute the relative percentile as a alternative measure to the relative hazard estimated in the Cox model for the difference in time to HCC between two groups; relative percentiles describe the relative increase or decrease in time to HCC between groups (17).

The relationships between intervention groups and biomarker levels were investigated using both simple one-way ANOVA models and regression methods that account for the correlation among repeated measurements (18). The latter methods were used to estimate and compare the mean of logarithmically transformed adduct levels among the intervention protocols at each of the six weekly time points. In addition, summary measures (peak and AUC) of adduct response over the entire observation period were computed for each animal. The average of these summary measures was calculated for each protocol and compared using standard ANOVA.

The repeated-measures regression model described above incorporates two parameters that describe the longitudinal tracking of aflatoxin-albumin adduct levels over time. Specifically, the correlation between two adduct measurements on any single rat taken $s$ weeks apart is assumed to be equal to $\gamma^s$. The parameter $\gamma$ represents the correlation between consecutive measurements, and the parameter $\theta$ represents how much the correlation increases or decreases as the time between measurements (lag) increases. Using this model, one can statistically test among several forms of the correlation structure, including whether the correlation can be summarized using a compound symmetry structure (which implies that the correlation is due to inherent animal effects) or an autoregressive structure (which implies that the correlation is due to direct dependence on prior outcomes). Parameter estimates were obtained using all data and separately for each intervention protocol.

All computations were done using either SAS version 6.11 (SAS, Inc., Cary, NC) or Splus (MathSoft, Seattle, WA).

**Results**

**Growth Rates of Rats.** The growth curves for the rats are shown in Fig. 1. All rats were treated with AFB$_1$ during weeks 8–13 of age. The overall growth rates of the rats fed oltipraz-supplemented diets in either the delayed-transient or persistent protocols were similar to that of the rats maintained on the control diet. Moreover, there were no significant differences in growth rates in the three experimental groups during the period of aflatoxin exposure and oltipraz interventions.
Effect of Treatment on Neoplasms. Apart from two early deaths due to anesthetic overdose with ether during blood collection, the first death in this study occurred during the 26th week, and the first death with a gross liver tumor was in the 67th week after initiation of aflatoxin dosing. Table 1 presents the effects of the oltipraz interventions on the incidence of AFBl-induced hepatic preneoplastic foci, adenomas, and HCC; however, only the most advanced hepatic lesion is reported for each rat. Foci were of course present in the livers of rats with adenomas or carcinomas. After 20 months, the 5-week exposure to AFBl produced a high incidence of HCC. There was a significant trend toward more HCCs in untreated animals (83%) relative to the delayed-transient intervention group (60%; Fisher's exact test, \( P = 0.028 \)) and persistent intervention group (48%; Fisher's exact test, \( P = 0.001 \)). Almost all of the tumor-bearing animals (32 of 34) in the no intervention group had multiple hepatic carcinomas, whereas in the delayed-transient and persistent intervention groups, significantly lower proportions of animals with multiple tumors were observed: 15 of 24 and 1 of 19, respectively. No differences in the histopathological characteristics of the HCCs were observed between the three groups. However, among the animals with no HCCs, there were no trends between treatment group and the occurrence of either foci or adenomas.

In addition to occurrence, the timing of HCC was significantly associated with treatment group. The estimated proportions free of HCC and free of multiple HCC events over the time from exposure using the censoring mechanisms described in the "Materials and Methods" section are shown in Fig. 2, A and B, respectively. Comparison of the Kaplan-Meier curves in Fig. 2A indicated that the animals in the persistent intervention group had marginally different HCC-free time than the delayed-transient group (\( P = 0.075 \)) but significantly longer HCC-free time than the no intervention group (\( P = 0.002 \)). Log-normal regression models estimated that the time to any HCC for the delayed-transient and persistent groups was 9 and 22% longer than the no intervention group \( (P = 0.000) \) but significantly longer HCC-free time than the no intervention group \( (P = 0.002) \). Log-normal regression models estimated that the time to any HCC for the delayed-transient and persistent groups was 9 and 22% longer than the no intervention group, respectively. For the time to multiple HCCs shown in Fig. 2B, Kaplan-Meier analyses indicated similar results when comparing delayed-transient and persistent groups (\( P = 0.055 \)), but both the delayed transient and the persistent groups had longer times free of multiple HCCs than the no intervention group \( (P < 0.001 \) and \( P = 0.019 \), respectively). Log-normal regression models estimated that the time to multiple HCC lesions for the delayed-transient and persistent groups was 15 and 32% longer than the no intervention group, respectively. A variety of extrabhepatic neoplasms were observed in these aged rats. The most common extrabhepatic neoplasm was MCL. The incidence of MCL was 39% in the no intervention group, and

### Table 1: Effect of oltipraz interventions on incidence of hepatic foci, adenomas, and HCCs

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No lesions</th>
<th>Foci</th>
<th>Adenomas</th>
<th>HCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>No intervention</td>
<td>1/40 (2.5)</td>
<td>2/40 (4.9)</td>
<td>24/41 (82.9)</td>
<td></td>
</tr>
<tr>
<td>Delayed-transient intervention</td>
<td>4/40 (10)</td>
<td>13/40 (32.5)</td>
<td>19/40 (47.5)</td>
<td></td>
</tr>
<tr>
<td>Persistent intervention</td>
<td>3/40 (7.5)</td>
<td>24/40* (60)</td>
<td>24/40* (60)</td>
<td></td>
</tr>
</tbody>
</table>

### Notes
- Male F344 rats were treated daily with 20 μg of AFBl, p.o., during weeks 8–12 of age and received either no intervention or delayed-transient or persistent interventions with 500 ppm oltipraz in the diet as detailed in "Materials and Methods."
- Only the most advanced hepatic lesion is reported for each rat.
- Significantly different from no intervention \((P < 0.05, \text{Fisher's exact test})\).
- Significantly different from delayed-transient intervention \((P < 0.05, \text{Fisher's exact test})\).
this incidence is typical for the F344 rat (19). The incidence of MCL decreased to 17.5% ($P = 0.048$) with the delayed-transient intervention with oltipraz and dropped even lower to 12.5% ($P = 0.010$) with the persistent intervention. The incidence of other extrahepatic neoplasms was low in all groups. No metastases were observed to the liver from other organs.

**Effect of Oltipraz Interventions on Biomarker Levels.** Shown in Fig. 3 is the time course for the formation of aflatoxin-albumin adducts in the serum of rats. Steady-state levels of approximately $350$ pmol of aflatoxin adducts/mg albumin were reached within 2–3 weeks of daily dosing with $20$ μg of AFB$_1$. These levels represent 1–2% of the administered dose of AFB$_1$. Levels of aflatoxin-albumin adducts declined slightly during the remaining period of AFB$_1$ exposure; however, they dropped rapidly once AFB$_1$ exposure was discontinued, reflecting the short half-life of circulating albumin in the rat. There were clear differences among treatment groups in the levels of biomarkers, as had been seen in earlier intervention studies with oltipraz (20). At each collection point during the aflatoxin exposure period, the group receiving the persistent intervention with oltipraz had significantly ($P < 0.0001$) lower biomarker levels than the control, no intervention group, such that steady-state levels were reduced by 50% to approximately $175$ pmol of aflatoxin adducts/mg albumin. The group receiving the delayed-transient intervention with oltipraz had biomarker levels that began to decline after the addition of oltipraz into the diet, such that they were intermediate between the control and persistent intervention groups after several weeks and converged with the levels of the persistent intervention group by the end of AFB$_1$ dosing. AUCs for the aflatoxin-albumin adducts over the entire aflatoxin exposure period decreased by 20 and 39% for the delayed-transient and persistent oltipraz interventions, respectively, compared to the no intervention group. There were linear trends ($P < 0.001$) among no, delayed-transient, and persistent intervention groups with respect to peak adduct levels (means: 434, 346, and 248 pmol/mg albumin, respectively) and AUC (means: 1321, 1012, and 740, respectively).

**Tracking of Biomarkers.** The serum samples that were collected weekly from the same animals during the period of exposure provided an opportunity to evaluate the degree to which biomarker levels tracked over time. An important observation was that there was little, if any, decrease in the correlation as the distance (lag) between the observations increased. This was confirmed in a formal analysis with the damped exponential correlation model that indicated that the $\theta$ parameter was not statistically significant from 0.0 when using either data from all three groups or when using data from each intervention group separately. Thus, the correlation between observations can be summarized by using a single intraclass correlation coefficient $\gamma$, which was estimated to be 0.296, and the 95% CI (0.205–0.368) indicated this was significantly greater than zero. There were no statistical differences in the estimate of $\gamma$ among the three intervention groups. The magnitude of this correlation is difficult to interpret absolutely; comparatively, higher correlation implies stronger predictability such that animals with high (low) adduct levels will tend to remain high (low).

**Relationship of Biomarker Levels to Group and Individual Cancer Outcomes.** Aflatoxin-albumin adduct measurements made at single time points and summary measures of adducts over time were used as covariates in a logistic regression model, predicting the incidence of HCCs. The variables showed a range in the strength of the association with carcinoma outcome, with AUC ($P = 0.010$) and peak adduct level ($P = 0.018$) showing the strongest associations. The parametric survival models fit with biomarker levels predicting the time to any or multiple HCCs showed similar trends in the direction and magnitude as with the logistic regression analysis (e.g., any HCC: AUC, $P = 0.009$, peak, $P = 0.010$). Hence, biomarker levels were significantly associated with both the occurrence and the timing of HCC.

Although statistically significant predictors of cancer incidence, neither AUC nor treatment group explained much of the variability seen in cancer outcomes. Using an $R^2$ measure of explained variability appropriate for binary outcomes (21), it is estimated that 5.7% of the variability in cancer outcomes was explained by AUC, and 6.5 and 13.9% of the variability in cancer outcomes was explained by the delayed-transient and persistent groups relative to the no intervention group, respectively.

The relationship of biomarker (AUC) and outcome (HCC) including observations from all three intervention groups is graphically depicted in Fig. 4A. It shows that the AUC was higher in animals developing HCCs than those that did not ($P = 0.01$). Fig. 4B shows the data in Fig. 4A stratified by treatment group. Previously described features of the oltipraz interventions can be seen in this figure; there is a downward trend of AUC with each of the interventions, and there is a downward trend of the proportion of HCCs (depicted by the relative distribution of white and non-white points) among intervention groups. These relationships fulfill the requirements for the intervention group to be a confounder for the AUC-HCC relationship. The distribution of AUC values for rats with either multiple or single HCC events substantially overlaps with those for HCC-free rats once the data are split by intervention group. Thus, there are no differences in the average AUC for those animals with no HCC versus any HCC when looking at the observations in each intervention group separately.

The quantitation of the ability of AUC to predict HCC incidence and time-to-HCC after adjusting for the relationship of HCC and intervention group is described in Table 2. The entries in the left columns of the table relate to predicting incidence of HCC using a logistic regression model, and the parameter that measures the strength of the association of HCC incidence and covariates is
the odds-ratio (as described in “Materials and Methods,” with values greater than 1 indicating higher odds of HCC). The entries in the right columns of the table relate to predicting HCC-free time using a log-normal regression model, and the parameter that measures the strength of the association of time to HCC and covariates is the relative percentile (with values greater than 1 indicating longer relative HCC-free time). The first several rows detail the univariate associations of incidence and time to HCC with AUC and intervention group. It can be seen that higher AUC is associated with higher incidence (\(P = 0.010\)) and shorter time to HCC (\(P = 0.009\)). Similarly, intervention groups have lower incidence and longer time to HCC than no intervention groups. When using all variables in a single multivariate model, the association of AUC decreases to nonsignificant levels, whereas differences still occur among intervention groups (significantly for the persistent group, but nonsignificantly for the delayed-transient group).

Discussion
The present results confirm and extend earlier results demonstrating the potent chemopreventive effect of oltipraz against AFB1-induced hepatocarcinogenesis in the rat. In the initial anticancer bioassay, the addition of 750 ppm of oltipraz to the diet throughout the period of carcinogen administration (25 \(\mu\)g of AFB1/rat/day, p.o., for 5 days/week over 2 weeks) afforded complete protection against the induction of HCC (11% versus 0%; Ref. 22). In the present study, in which a lower dose of chemopreventive agent (500 ppm of oltipraz) and a 3-fold higher cumulative exposure to AFB1 was used (20 \(\mu\)g of AFB1/rat/day for 35 days), the persistent administration of oltipraz throughout AFB1 exposure significantly reduced the incidence of HCC (83 to 48%, \(P < 0.001\)). Although experimentally expedient, protocols such as these in which administration of the chemopreventive agent subsumes carcinogen exposure do not represent an accurate paradigm for interventions in human populations, where lifelong exposures to carcinogens may occur yet opportunities for chemoprevention may be more limited. Thus, an additional intervention schedule was used in these studies as a model for human interventions, in which the administration of oltipraz was delayed and transient relative to aflatoxin exposure. Such a schedule mimics Phase II/III clinical trials. In this instance, despite the disadvantageous bias, a significant reduction in the incidence of HCC was observed (83 to 60%, \(P = 0.028\)) when oltipraz was fed for 2 weeks in the midst of a 5-week exposure to AFB1. This chemopreventive
efficacy of a delayed, transient intervention with oltipraz was predicted based upon the substantive reduction in the hepatic burden of GST-P positive foci, a presumptive preneoplastic lesion, reported earlier (23). Interestingly, both intervention protocols engendered significant delays in the development of single HCCs or the amplification of single to multiple HCCs when neoplasms occurred.

A significant decrease in hematopoietic neoplasia was also observed with oltipraz interventions. MCL is a common neoplasm in the F344 rat that is not associated with exposure to AFB1 (19). Roebuck et al. (22) initially reported that feeding 750 ppm of oltipraz significantly reduced the incidence of MCL from 53 to 30%. In the current study, the incidence of MCL was reduced from 39 to 18% and 13%, respectively, by the delayed-transient and persistent interventions with 500 ppm of oltipraz. Thus, oltipraz appears to block one or more early processes of MCL development. Intriguingly, Rao et al. (24) have recently reported that oltipraz inhibits the formation of another hematopoietic neoplasm, thymic lymphosarcoma, in rats treated with the heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). With multiple reports of oltipraz preventing hematopoietic tumors and the paucity of approaches for the prevention of such tumors, oltipraz may present a unique opportunity for chemoprevention in this setting.

A heretofore neglected opportunity in the development and use of experimental models of cancer chemoprevention is the ability to examine the predictive significance of intermediate biomarkers to the cancer process. Such models afford the opportunity to modulate risk of cancer while maintaining carcinogen exposure constant. A particularly promising class of markers are those that measure biologically effective dose (4). These biomarkers reflect the internal dose at the molecular target (or a reasonable surrogate thereof) and are usually measured as DNA-adducts or protein-adducts. Numerous epidemiological and experimental studies have established dose-response associations between external exposures and adduct levels. Correlations have also been observed with chemopreventive regimens in parallel cohorts of animals between the degree of inhibition of tumorigenesis and the extent of reductions in DNA-adduct and/or protein-adduct levels. Collectively, these correlative linkages between biomarkers and reduced risk of cancer outcomes suggest that adduct biomarkers might serve: (a) as short-term end points for assessing the efficacy of chemopreventive agents; and (b) as tools for personalized risk assessments.

Albumin is quantitatively the most abundant target for aflatoxin, and aflatoxin-lysine has been identified as the major adduct in rat and human albumin (25). This adduct is formed by the same metabolic pathway that leads to aflatoxin-DNA adduct formation. Indeed, a constant relationship between levels of aflatoxin binding to serum albumin and hepatic DNA have been observed in several studies in experimental animals (26, 27). Levels of aflatoxin-albumin adducts also qualitatively reflect the relative susceptibility of different species to aflatoxin-induced hepatocarcinogenesis (28). Moreover, several studies have demonstrated that levels of aflatoxin-albumin adducts can be modulated by a variety of chemopreventive interventions with oltipraz and its unsubstituted congener, 1,2-dithiole-3-thione (20, 29). The repetitive measurements of aflatoxin-albumin adducts performed during this bioassay reflect, quantitatively and qualitatively, the adduct patterns determined earlier from serum samples obtained from animals treated with identical aflatoxin/oltipraz regimens, but using daily serial sacrifices of animals for measurements of multiple intermediate end points (20). Independent analytical methodologies were used for the aflatoxin-albumin adduct measurements in these two studies. AUC measurements in both studies revealed approximately 20 and 40% reductions in aflatoxin-albumin adduct burdens throughout the AFB1 exposure period with the delayed-transient and persistent oltipraz interventions, respectively. Strikingly, comparable reductions were seen in the final incidence of HCC with the two oltipraz interventions in the present study. As a consequence, there was a strong association between biomarker burden (AUC) and carcinoma outcome (P = 0.01). Another notable feature was the significant intraclass correlation (tracking) between sequential biomarker measurements. These studies, therefore, directly confirm the usefulness of monitoring levels of the aflatoxin-albumin adduct in population-based approaches to evaluate pharmacodynamic action in interventions with oltipraz and similarly acting agents. Indeed, this biomarker is currently being used to assess the efficacy of oltipraz in humans at high risk for aflatoxin exposure and development of HCC in rural China (30). Measurements of albumin adducts appear to be particularly useful because they are by simple, facile immunoassays that can be applied to large numbers of samples in field studies.

However, the apparent strength of the observed association with biomarker burden and cancer outcome reflects the proportional distribution of cases and non-cases of cancer within the no intervention and persistent intervention arms. Thus, very strong associations will be seen when protective efficacy is maximized through judicious selection of dose and duration of carcinogen and anticarcinogen (29, 31). As one example, Hecht et al. (32) have shown complete segregation of the distribution of 4-(methylisocya- nosamo)-1-(3-pyridyl)-1-butane-derived hemoglobin adducts under dosing conditions in which 4-(methylisocyanamo)-1-(3-pyridyl)-1-butane alone induced lung tumors in 70% of the rats, whereas an intervention with phenethylisothiocyanate reduced the tumor incidence to background. In the present study, the two intervention protocols with oltipraz produced roughly equivalent distributions of HCC cases and controls within each intervention arm and afforded the opportunity to examine the extent to which there exists a prospective link between a molecular biomarker of the biologically effective dose of aflatoxin (i.e., aflatoxin albumin adduct) and HCC outcome in individual animals. However, in this stringent setting, the biomarker provided no assistance in discriminating between individual rats destined to develop HCC and those that did not. Given the complex interactive nature of the carcinogenic process, it is perhaps unreasonable to expect a single, early marker to predict cancer outcomes. Production of genetic damage by hepatocarcinogens is not a sine qua non for cancer. For example, the genotoxic component of 2-acetylaminofluorene has been recently suggested to play a minor role in its hepatocarcinogenicity (33). Many other factors, including recurrent cytotoxicity, cell proliferation, and nutritional status, can exert strong postinitiation effects to either enhance or retard tumorigenesis. Sensitive, non-invasive biomarkers or combinations of markers for these processes need to be developed and applied. An additional confounding factor relates to the strength of the surrogacy of the aflatoxin-albumin adduct for genotoxicity. Although there appears to be good correspondence between total aflatoxin DNA and protein-adduct burden following aflatoxin exposure, reflecting the common metabolic origin of these adducts, the relationship between DNA adduct burden and the effective mutational target within the genome may be less precise. The effective burden of genomic damage is likely to directly influence tumor incidence and multiplicity. Such correlative degeneracy may reflect the important role that DNA sequence context plays in aflatoxin mutagenesis as well as the impact of differential rates of repair for damage to critical and noncritical target genes (34). Lastly, it should be noted that AFB1 exposure levels (and hence, biomarker levels) used in this rodent experiment were considerably higher than found in human populations. Whether stronger relationships between biomarker...
and outcomes might be observed at lower exposure levels remain to be established.

The overall strategy to use chemical-specific biomarkers as intermediate end points has a wide range of potential, largely unexplored applications for the refinement of risk quantitation. In the carcinogen testing arena, determinations of these types of early markers could accelerate predictions of chronic toxicity and cancer outcomes well before the traditional 2–3-year time points. Furthermore, these mechanistically based biomarkers might become efficient tools with which to rationally compare new transgenic animal models with standard animal bioassay species. Such applications are needed as the number and diversity of chemicals to be tested increase. Similarly, these biomarkers can be used to assess alterations in risk following chemopreventive interventions. In our study, strong concordance is observed between the reduction in risk of HCC and diminution of biomarker levels following treatment with oltipraz. Groupwide, population-based changes in biomarker levels are readily detected as a function of intervention status. However, our results also point to strong limitations in the interpretation of carcinogen-adduct biomarker measurements. These adducts are unlikely to serve as unimodal measures of personal risk. Although changes in relative risk can be readily discerned, these markers do not appear suitable for absolute prediction of disease outcome. As a consequence, the use of adduct biomarkers in quantitative risk assessment should be pursued cautiously.

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