Complete Protection against Aflatoxin B₁-Induced Liver Cancer with a Triterpenoid: DNA Adduct Dosimetry, Molecular Signature, and Genotoxicity Threshold

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Abstract

In experimental animals and humans, aflatoxin B₁ (AFB₁) is a potent hepatic toxin and carcinogen. The synthetic oleanane triterpenoid 1-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Im), a powerful activator of Keap1-Nrf2 signaling, protects against AFB₁-induced toxicity and preneoplastic lesion formation (GST-P-positive foci). This study assessed and mechanistically characterized the chemoprotective efficacy of CDDO-Im against AFB₁-induced hepatocellular carcinoma (HCC). A lifetime cancer bioassay was undertaken in F344 rats dosed with AFB₁ (200 μg/kg rat/day) for four weeks and receiving either vehicle or CDDO-Im (three times weekly), one week before and throughout the exposure period. Weekly, 24-hour urine samples were collected for analysis of AFB₁ metabolites. In a subset of rats, livers were analyzed for GST-P foci. The comparative response of a toxicogenomic RNA expression signature for AFB₁ was examined. CDDO-Im completely protected (0/20) against AFB₁-induced liver cancer compared with a 96% incidence (22/23) observed in the AFB₁ group. With CDDO-Im treatment, integrated level of urinary AFB₁-N7-guanine was significantly reduced (66%) and aflatoxin-N-acetylcysteine, a detoxication product, was consistently elevated (300%) after the first AFB₁ dose. In AFB₁-treated rats, the hepatic burden of GST-P-positive foci increased substantially (0%–13.8%) over the four weeks, but was largely absent with CDDO-Im intervention. The toxicogenomic RNA expression signature characteristic of AFB₁ was absent in the AFB₁ + CDDO-Im–treated rats. The remarkable efficacy of CDDO-Im as an anticarcinogen is established even in the face of a significant aflatoxin adduct burden. Consequently, the absence of cancer requires a concept of a threshold for DNA damage for cancer development. Cancer Prev Res; 7(7); 658–65. ©2014 AACR.

Introduction

Hepatocellular carcinoma (HCC), the most common form of primary liver cancer, is the third leading cause of cancer mortality worldwide (1). It has been estimated that between 4.6% and 28.2% of all HCC cases worldwide may be attributed to aflatoxin exposure (2). Thus, a considerable research effort has focused on prevention strategies to reduce the impact of aflatoxin-induced HCC, including postharvest mitigation of food contamination by the mold Aspergillus flavus that forms aflatoxin (3) and dietary change to foodstuffs less prone to fungal contaminations (4). The use of chemical or dietary interventions to block, retard, or reverse carcinogenesis, a strategy termed chemoprevention, represents another promising approach for the reduction of HCC. Initial cancer prevention bioassays in aflatoxin-treated rats utilizing the antischistosomal drug oltipraz demonstrated reductions in the incidence of HCC from 20% to 0% when low doses of aflatoxin B₁ (AFB₁) were used (5) and from 83% to 48% when a higher total dose of AFB₁ and a longer duration of dosing was used (6). In both cases, significant, but incomplete reductions in levels of hepatic aflatoxin-derived DNA adducts were observed in parallel cohorts of animals. Oltipraz was subsequently used in randomized, placebo-controlled phase II chemoprevention trials in an aflatoxin-endemic region of China, where pharmacodynamic action indicative of enhanced detoxication of aflatoxin was reported (7, 8).

More recently, the synthetic oleanane triterpenoid 1-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Im) has been shown to inhibit aflatoxin-induced tumorigenesis in the rat as evidenced by a significant...
reduction in hepatic focal burden of glutathione S-transferase placental form (GST-P–positive foci) preneoplastic lesions (9). Notably, CDDO-Im was nearly 100 times more potent than oltipraz in this short-term in vivo model. Mechanistic studies illustrated that CDDO-Im is an exceptionally potent activator of Keap1-Nrf2 signaling (10, 11), which leads to enhanced conjugation of the 8,9-epoxide of AFB1 with glutathione through the actions of glutathione S-transferases (GST) and consequent diminution of DNA adducts formed from this ultimate carcinogenic electrophile. On the basis of this unparalleled potency of CDDO-Im, we used a subchronic aflatoxin dosing regimen in a lifetime bioassay in F344 rats to assess protective efficacy against hepatocarcinogenesis. Serial urine collections during dosing afforded the opportunity to assess the impact of intervention on AFB1 genotoxicity and detoxication. The model was also used to assess whether a short-term toxicogenomic signature of aflatoxin hepatocarcinogenicity could predict the extent of risk reduction by the intervention.

Materials and Methods

Chemicals

AFB1 was purchased from Sigma-Aldrich. CDDO-Im was synthesized as previously described (12).

Animals

Male F344/NHsd rats were purchased from Harlan and housed under controlled conditions of temperature, humidity, and lighting. Animals were fed AIN-76A purified diet (Teklad) without the addition of the dietary antioxidant ethoxyquin. Food and water were available ad libitum. Fresh diet was provided to animals at least twice per week. Rats were weighed daily during the dosing period and twice weekly thereafter. All experiments were approved by The Johns Hopkins University (Baltimore, MD) Animal Care and Use Committee.

Treatment protocol for protection against hepatocarcinogenesis

Rats were acclimated to diet and housing conditions for 1 week. Figure 1 outlines the treatment schedule. At 5 weeks of age and approximately 85 g body weight, the rats were randomly assigned to the two treatment groups: AFB1 + vehicle (henceforth, called AFB1) or AFB1 + CDDO-Im. The rats were gavaged with either 30 µmol (16.2 mg)/kg rat with CDDO-Im or a vehicle of 10% dimethyl sulfoxide, 10% Cremophor-EL, and PBS, three times per week for 5 successive weeks. This vehicle has been used previously with CDDO-Im (9, 11). Beginning at week 6 and 2 hours before CDDO-Im treatment, all rats received 200 µg AFB1 dissolved in dimethyl sulfoxide/kg body weight via oral gavage daily for 4 weeks. Following the first dose of AFB1 and at weekly intervals during AFB1 treatment, all rats were housed in glass metabolic cages and urine was collected on ice for a period of 24 hours.

Analysis of cancer

All rats were euthanized and necropsied when clinical observations indicated that the rat was in pain or would likely not survive longer than 12 hours. The latter criteria were substantial (>15%) and rapid loss of body weight, failure to groom, and/or inability to ambulate. Standardized sections of normal hepatic tissue and all abnormal tissues, including all hepatic tumors, were fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin. Hepatic histopathological analyses were performed according to the criteria of Eustis and colleagues (13).

Analysis of aflatoxin metabolites in urine

Immediately following the urine collection, samples were centrifuged at 150 × g and adjusted to an acidic pH using 0.5 mol/L ascorbic acid. Urines were analyzed for levels of aflatoxin-N7-guanine and aflatoxin-N-acetylcysteine by isotope dilution mass spectrometry (14). Levels were normalized to creatinine content as measured using a spectrophotometric creatinine kit (Eagle Diagnostics, Inc.).

Treatment for hepatic aflatoxin-DNA adducts, hepatic foci, and gene signature analysis

To analyze liver tissue following AFB1 exposure with or without CDDO-Im treatment, a subset of rats were treated as in Fig. 1 and serially sacrificed 24 hours following the last AFB1 dose at the end of each week (i.e., weeks 7, 8, 9, and 10 of age). Multiple 2-mm thick sections were cut from the left lateral, median, and right lobes of the liver and fixed in 4°C acetone, stained for expression of GST-P–positive foci, and analyzed by light microscopy. The observed focal data of number of foci per unit tissue area and their focal transac- tion were subjected to morphometric transformation resulting in the volume percent of liver occupied by GST-P–positive foci (15, 16). Within minutes of sacrifice, the remaining liver was flash frozen in liquid nitrogen and stored at −80°C. DNA was isolated (17) and analyzed for levels of aflatoxin-DNA adducts by isotope dilution mass spectrometry. Total DNA content was determined spectrophotometrically using diphenylamine. Total RNA was isolated from frozen liver tissue (18). The levels of RNA for all genes were analyzed by quantitative real-time PCR. Each gene was normalized to that of β-actin and the relative value for the control samples was set at one. The genes analyzed were based on a transcriptome signature shown to be predictive of aflatoxin hepatocarcinogenesis (19–21).
Statistical analyses

Body weights and levels of biomarkers were compared between groups using the Student t test. Kaplan–Meier curves were compared by the log-rank test.

Results

Growth of rats and AFB1 toxicity

Figure 2 shows the growth inhibitory effects of AFB1 and amelioration of this persistent toxicity by concurrent treatment with CDDO-Im. Within one week, the weight gain of the AFB1-treated group was significantly diminished (P < 0.001) compared with the AFB1 + CDDO-Im group (Fig. 2, insert). Although this impact on growth somewhat subsided with termination of daily AFB1 exposure at week 10, the diminished body weight of unprotected, early in life AFB1 treatment persisted throughout their lifetime.

Lifetime cancer bioassay

Profound differences in HCC incidence were seen between treatment groups. In the AFB1 group, the first HCC appeared in a rat that died at 44 weeks of age. The incidence of HCC in the AFB1 group was 96% (22 of 23 rats) with the majority of the rats presenting with multiple HCCs that often appeared to coalesce into one larger tumor mass obliterating the normal gross hepatic architecture. The one animal in the AFB1 group that was not diagnosed with HCC had five large preneoplastic foci with an average focal transactional diameter of 1.3 mm when it died, relatively young, at 35 weeks of age. The histopathological picture was radically different in the AFB1 + CDDO-Im group as no HCCs were found. The most advanced AFB1-related lesions were putative preneoplastic foci detected in only 3 of the 20 rats. These 3 rats died at 93, 95, and 111 weeks of age and the foci were less than half the size of foci seen in the AFB1 -treated rat that died at 35 weeks of age.

The median age of death in the AFB1 group was 74 weeks compared with the AFB1 + CDDO-Im group median of 90 weeks (P < 0.01). The overwhelmingly positive impact of CDDO-Im on prevention of HCC and ultimately survival against a large exposure to AFB1 is shown in Fig 3. Aged control F344 rats largely die of mononuclear cell leukemia (22) or marked bilateral chronic progressive nephropathy (23). In the AFB1 + CDDO-Im group, the incidence of nephropathy was 75% (15/20) and leukemia was 50% (10/20) with the majority having both pathologies. In the AFB1 group, the prevalence of these lesions was much lower: 17% (4/23) had nephropathy and 30% (7/23) had leukemia. Because the rats treated with CDDO-Im lived longer and free of HCC, they then became susceptible to the common chronic diseases of age in this rat strain. The burden of HCC was almost certainly the major contributing factor to the clinical decline and death of these AFB1 rats rather than these comorbidities.

AFB1 DNA adducts and mercapturic acid

Urinary biomarkers of aflatoxin were measured in the lifetime bioassay rats during the 28-day AFB1-treatment period to determine relationships with protection by CDDO-Im. All animals had biomarker levels well above the analytical limit of determination. Figure 4A depicts the urinary excretion of aflatoxin-N7-guanine, a biomarker of the biologic effective dose of AFB1. Also shown is the average administered daily dose of AFB1. The AFB1 + CDDO-Im rats excreted significantly less aflatoxin-N7-guanine than the AFB1 rats (overall, 34% of the AFB1 group) despite receiving a cumulative 20% higher dose of AFB1. At the end of the last week of the dosing, the AFB1 rats had more than seven times as much aflatoxin-N7-guanine per day in urine as did the protected group.

A major route of detoxication of AFB1 is through conjugation of the aflatoxin-8,9-epoxide with glutathione by GSTs and ultimately, excretion in the urine as a mercapturic acid. Elimination of aflatoxin-N-acetylcysteine in the AFB1 + CDDO-Im rats was initially 3-fold higher than in the AFB1 group and remained at this elevated rate for the duration of the dosing period (Fig. 4B). An adaptive response in the AFB1 rats was an increase in aflatoxin-N-acetylcysteine.
formation and elimination beginning in the second week of dosing and thereafter increasing to levels greater than in the AFB1 + CDDO-Im rats.

In a separate cohort of animals treated identically, livers were collected 24 hours post-AFB1, at weekly intervals over 4 weeks and were analyzed for the hepatic burden of aflatoxin-DNA adducts. The major and stable aflatoxin-N7-guanine–derived adduct in liver is 8,9-dihydro-8-(2,6-diamino-4-oxo-3,4-dihydropyrimid-5-yl formamido)-9-hydroxyaflatoxin B1 (FAPyr). As shown in Fig. 5, hepatic FAPyr levels remained 2- to 3-fold higher in the AFB1 group than in the AFB1 + CDDO-Im group. Although initially the primary adduct formed is aflatoxin-N7-guanine, by 24 hours aflatoxin-N7-guanine has either undergone repair, depurinated, or ring-opened to form FAPyr. Overall, aflatoxin-N7-guanine adducts were 53% lower in the AFB1 + CDDO-Im group, foci were totally absent at 8 and 15 days, and at 22 and 28 days, the focal volume percentage was 0.02 and 0.01, respectively. Only one animal in each of the day 22 and 28 groups had any foci; whereas, all AFB1-treated rats had multiple foci from day 15 onward. Clearly, the CDDO-Im intervention largely prevented the formation of these putative preneoplastic, microscopic foci commonly seen after aflatoxin exposure.

**Validation of an AFB1 gene signature**

Merrick and colleagues (20) defined a discrete 14 gene signature derived from transcript profiling that predicted hepatocarcinogenic responses to subchronic AFB1 exposure (19). These genes, Wwex, Fhit, Adam8, C8orf46 homolog, Myb2, Abcb1b, Cdh13, Ddit4l, Akr7a2, Akr7a3, Abcc3, Cxcl1, Gsta5, and Grin2c, represent cell-cycle progression, DNA damage response and xenosensor, and detoxication pathways. However, in a separate experiment, we observed that treatment of rats with a single dose of 30 μmol CDDO-Im/kg body weight by itself led to the induction of transcripts for Myb2, Ddit4l, Abcc3, Gsta5, and Akr7a1 (Akr7a2 and Akr7a3 are actually human genes) and a decrease in Cxcl1 independent of AFB1 (data not shown). After censoring for these genes, we examined the effect of the AFB1 + CDDO-Im intervention on the expression of the remaining seven AFB1 signature genes in AFB1-treated rats compared with vehicle was entirely consistent with the literature report (20). Moreover, the intervention with CDDO-Im almost completely abrogated the AFB1-induced changes in RNA expression. Five (Wwex, Fhit, Adam8, Cdh13, and Grin2c) of the seven genes were expressed in the protected group at
levels no different than measured in the livers of control animals not receiving AFB1 and expression of the other two, \textit{Apc1b1} and \textit{C8orf46}, declined 67\% and 75\%, respectively, toward values of untreated animals.

\textbf{Discussion}

The inclusion of risk-reduction cohorts in animal bioassays allows not only the evaluation of novel preventive agents, but also provides a unique perspective to assess possible genotoxicity thresholds and to validate predictive molecular signatures of carcinogenesis. This study demonstrates the remarkable efficacy and extreme potency of a synthetic oleanane triterpenoid as an inhibitor of carcinogenesis, in this instance, induced by a known human carcinogen. The complete ablation of HCC development coupled with extended survivorship by CDDO-Im in a model in which aflatoxin induces a 96\% incidence of HCC is unparalleled, irrespective of the dose or chemical class of chemopreventive agent used (24). Decades of mechanistic studies on aflatoxin hepatocarcinogenesis in rats provide a clear perspective on the roles of carcinogen metabolism, DNA damage, and hepatotoxicity on this pathogenesis (7). Previous studies by our group have indicated that the protection provided by CDDO-Im in this model is achieved largely through interaction with signaling pathways mediated by the transcription factor Nrf2 (9, 11). Hepatic expression of Nrf2 target genes known to be involved in aflatoxin detoxification, namely, aldo-keto reductase 7A1 and GSTs, is elevated by CDDO-Im. The present results in which hepatic and urinary levels of aflatoxin-DNA damage products have revealed that the FAPyr adduct is about ten times more mutagenic than the aflatoxin-\textit{N}7-guanine and aflatoxin N-acetylcysteine increase substantially. Also at this time, the percentage of hepatocytes expressing the presumptive preneoplastic phenotype of GST-P positivity dramatically rises from a fraction of a percentage to more than 10\%. These foci typically harbor an increased capacity to detoxify carcinogens (28) and likely account for the increased excretion of aflatoxin-N-acetylcysteine to levels even higher than those induced by CDDO-Im. Indeed, evidence of resistance to AFB1 cytotoxicity in AFB1-induced preneoplastic lesions has been associated with increased glutathione levels and GST activity (29). The marked elevation in excretion of aflatoxin-\textit{N}7-guanine may reflect the increased apoptosis observed in the residual hepatocytes of the AFB1-treated animals at these later points in the dosing regimen (30). Aflatoxin-induced DNA damage is a key mechanistic step in the induction of HCC. The major DNA adducts produced by aflatoxin are aflatoxin-\textit{N}7-guanine and its stable DNA oxidation product aflatoxin-FAPyr (31). Studies of the mutational potency of these two DNA damage adducts have revealed that the FAPyr adduct is about ten times more mutagenic than the aflatoxin-\textit{N}7-guanine adduct, which in turn is a more toxic lesion (32, 33). Analysis of the DNA adducts in our investigation reveals that at 24 hours postdosing, the FAPyr lesion already precedes the AFB1-treated animals at these later points in the dosing regimen (30). Aflatoxin-induced DNA damage is a key mechanistic step in the induction of HCC. The major DNA adducts produced by aflatoxin are aflatoxin-\textit{N}7-guanine and its stable DNA oxidation product aflatoxin-FAPyr (31). Studies of the mutational potency of these two DNA damage adducts have revealed that the FAPyr adduct is about ten times more mutagenic than the aflatoxin-\textit{N}7-guanine adduct, which in turn is a more toxic lesion (32, 33). Analysis of the DNA adducts in our investigation reveals that at 24 hours postdosing, the FAPyr lesion already precedes.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Days of AFB1</th>
<th>Number of rats</th>
<th>Focal volume, mean (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0(^a)</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>AFB1</td>
<td>8</td>
<td>3</td>
<td>0.01 (0–0.04)</td>
</tr>
<tr>
<td>15</td>
<td>3</td>
<td>0.25 (0.10–0.34)</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>3</td>
<td>3.22 (1.89–5.86)</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>6</td>
<td>13.81 (5.25–23.11)</td>
<td></td>
</tr>
<tr>
<td>AFB1+CDDO-Im</td>
<td>8</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>3</td>
<td>0.02 (0–0.064)</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>4</td>
<td>0.01 (0–0.02)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Sacrificed on day 28.
agents exhibit linear dose–response curves for the formation of covalent adducts, and thereby no "safe level" or threshold dose exists. Recent literature (35) has challenged this default "no-threshold" assumption, demonstrating that direct acting alkylating agents such as ethyl methanesulfonate and methyl- and ethyl nitrosourea do not follow a linear pattern at low-dose levels. Mechanistic studies show that these low-dose exposures may be influenced by homeostatic mechanisms, such as repair by DNA methyltransferases (36, 37). Aflatoxin acts as a classic genotoxin characteristically binding to the N7 atom of guanine (38). Previous studies conducted in the F344 rat have shown that AFB1 observes a linear dose–response curve in regards to macromolecular adduct formation over a wide range (39–43) extending to doses as low as 0.16 ng/kg (44). Consequently, it has been concluded that a "no threshold" paradigm exists. By combining tumor incidence data from long-term rat and trout studies, Bechtel (45) extended this observation to support that hepatic cancer risk is linearly proportional to hepatic aflatoxin DNA adduct concentration. However, our data lead to a different conclusion, pointing to a view that substantial aflatoxin-DNA damage is not sufficient for development of HCC. Moreover, the absence of cancer in this experimental setting supports the concept of a threshold for biologic mode of action that links DNA damage to the development of HCC. Kaden and colleagues (46) demonstrated an apparent saturation of mutation induced by AFB1 despite a linear increase in the amount of aflatoxin-DNA adducts formed in human lymphoblast cells. They hypothesized the presence of an inducible error-free DNA repair system at higher levels of adduct burden. Clearly, although DNA adducts may lead to mutations, an adduct is not the equivalent of a mutation. Smela and colleagues (33) have reported that aflatoxin-N7-guanine requires 30 lesions per induced mutation, whereas FAPyr is 10-fold more mutagenic and 35% of FAPyr adducts result in mutation.
Additional evidence that CDDO-Im is altering the carcinogenic mode of action at steps beyond adduct formation comes from measurements of a predictive genomic signature of carcinogenicity. Current rodent bioassays are time consuming and very expensive, and are incapable of evaluating the high numbers of existing and new chemicals awaiting testing. Molecular expression analyses are being undertaken to predict the carcinogenicity of untested chemicals using relatively inexpensive, high-throughput platforms (47). Such gene expression-based predictive models have been shown to be effective tools for identifying hepatocarcinogens (19). Transcript profiles following subacute and chronic dosing regimens with a range of rat hepatocarcinogens, including AFB1, were used to derive predictive computational models for their classification before tumor development. Merrick and colleagues (20, 21) confirmed this AFB1 gene signature in a 90-day feeding study (1 ppm AFB1) using microarray, RNA-Seq, and qPCR methods. These genes included the disintegrin metalloprotease Adam8, the drug transporter Abe1b1, the DNA damage-sensitive C8orf46 homolog as well as genes that are repressed after genotoxic challenge (Waxw and Fhit; ref. 19). Results from our 28-day, high-dose AFB1 study completely disappeared in the context of our risk ablation intervention with CDDO-Im. Thus, these gene expression changes seem to accurately reflect the underlying biology that drives the liver toward HCC.

Collectively, this lifetime bioassay with the human carcinogen AFB1, coupled with an intervention with the synthetic oleanane triterpenoid CDDO-Im, (i) establishes the remarkable efficacy of CDDO-Im as an anticarcinogen, (ii) defines operationally the presence of a genotoxicity threshold in this carcinogenesis model, and (iii) provides an additional, unique element for the validation of a predictive molecular signature of hepatocarcinogens.

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disclosure of potential conflicts of interest

M.B. Sporn has a commercial research grant from Reata Pharmaceuticals ownership interests including patents on triterpenoids. No potential conflicts of interest were disclosed by the other authors.

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