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Cancer Prevention Research

Perspective

See related article by Johnson et al., p. 658

Of Mice, Rats, and Men: Could Nrf2 Activation Protect against Aflatoxin Heptocarcinogenesis in Humans?

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Abstract

In this issue, Johnson and colleagues provide a remarkable demonstration of the potential for "chemoprevention" of cancer from mutagenic chemicals. The authors demonstrated complete protection of rats from a carcinogenic treatment regimen with the potent dietary mutagen and hepatocarcinogen, aflatoxin B_1 (AFB) by pretreatment with a synthetic oleanane triterpenoid, 1-[2-cyano-3-,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Im). This study is notable for two reasons: (i) Activation of the Nrf2/Keap1/ARE "antioxidant response" pathway by CDDO-Im conferred complete protection against AFB-induced hepatocellular carcinomas in the Fisher F344 rat (a strain frequently used in life-time carcinogenicity bioassays), and (ii) extensive AFB–DNA adduct formation was seen in all animals at early time points, including those treated with CDDO-Im, *albeit* at lower levels (~30% of the untreated animals), suggesting a strong divergence in the association between early DNA-damaging events, and tumor formation later in life. The authors suggest that this provides compelling experimental support for the concept of carcinogenic "thresholds" for mutagenic chemicals, because the treatment reduced persistent, mutagenic adducts (AFB–FAPyr adducts) only by 70%, but nearly completely eliminated tumors after approximately 2 years and preneoplastic lesions 6 weeks after the last dose of AFB. *Cancer Prev Res; 7(7); 653–7. ©2014 AACR.*

This work is an extension of numerous previous studies by the laboratories of Roebuck, Groopman, and Kensler, who have over the years demonstrated that aflatoxin B_1 (AFB) is both a remarkably potent hepatocarcinogen in laboratory rats and that carcinogenesis and mutagenesis of AFB can be readily modulated by "natural" as well as synthetic activators of the antioxidant/detoxification or Nrf2/Keap1/ARE response (2). Building on the pioneering work of Paul Talalay at Johns Hopkins University (Baltimore, MD; ref. 3), Kensler and colleagues and others previously demonstrated that a variety of synthetic and naturally occurring chemicals can modulate this endogenous signaling pathway by perturbing intracellular redox homeostasis (reviewed in ref. 4). Under basal conditions, Keap1 associates with the Nrf2 protein, signaling for its polyubiquitination and subsequent proteasomal degradation (5). However, in response to an increase in the intracellular oxidation state, specific redox-sensitive cysteine residues on Keap1 are oxidized, and subsequently, Keap1 association with Nrf2 is modified, resulting in a saturation of Keap1 ubiquination signaling, thereby allowing newly synthesized Nrf2 to translocate to the nucleus, in which it dimerizes with members of the small Maf family and binds to a specific *cis*-acting DNA-binding motif deemed the "antioxidant response element" or ARE (5). Numerous genes involved in phase II biotransformation, such as those involved in glutathione biosynthesis, as well as other stress response proteins, are upregulated via this mechanism.

In this study, Johnson and colleagues pretreated Fisher F344 rats, a strain that is highly susceptible to the hepatocarcinogenic effects of AFB, with the potent Nrf2 activator 1-[2-cyano-3-,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Im) for 5 weeks (three doses of 30 μmol/L by oral gavage, every other day) before administration of AFB and then concurrently with daily AFB (200 μg/kg by gavage) for 4 weeks beginning at week 6. Urine was collected for analysis of the presence of AFB–N^7-guanine, indicative of DNA damage and repair. A subset of animals was sacrificed 28 days after AFB treatment and evaluated for the presence of preneoplastic foci. But 20 of the CDDO-Im + AFB and 22 of 23 of the vehicle control + AFB were maintained until they died from natural or treatment-related causes, up to 100 weeks. A Kaplan–Meier mortality curve provided dramatic evidence of the effectiveness of the CDDO-Im treatment. Ninety-six percent (22 of 23) of the animals treated with AFB but not administered CDDO-Im either died from hepatocellular carcinoma or had extensive carcinomas in their livers at termination, whereas none of the 20 CDDO-Im–treated animals developed hepatocellular carcinoma. The strikingly clear results might suggest that activating ligands of the Nrf2 antioxidant response pathway in humans could serve as an effective "chemointervention" approach for human

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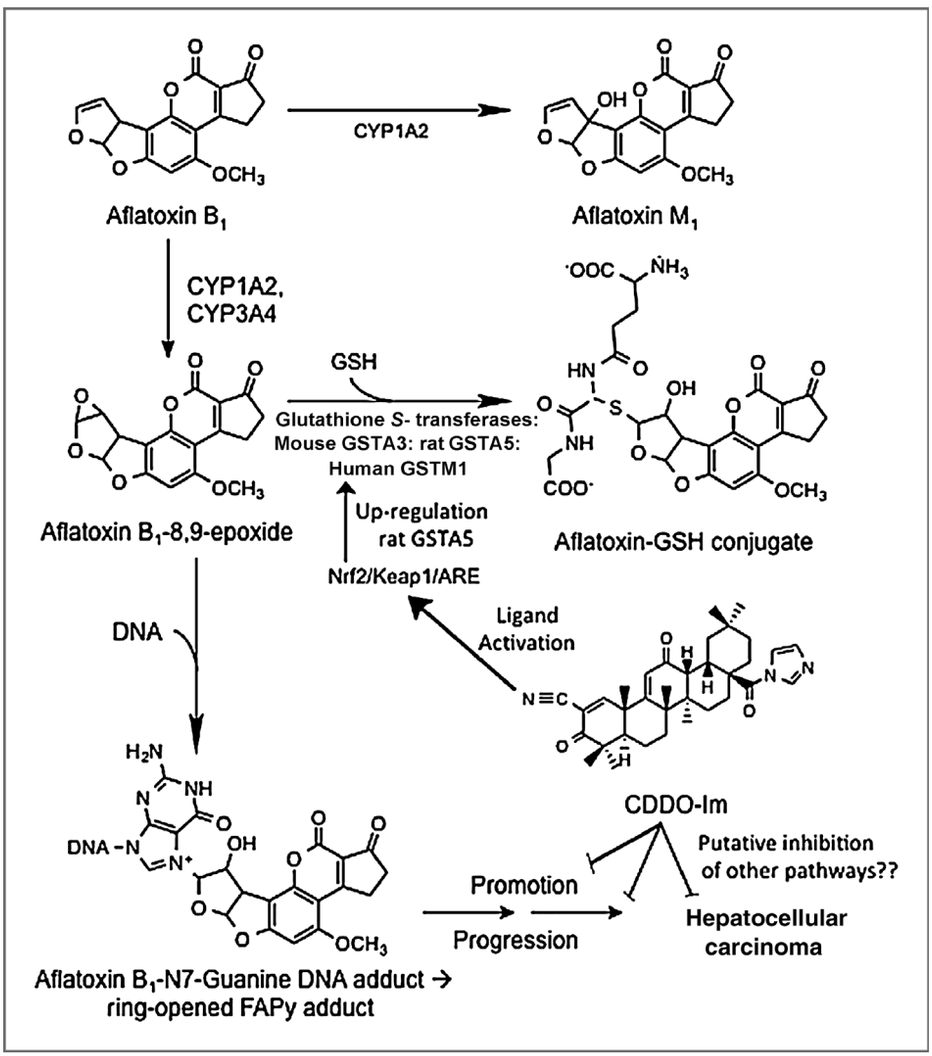

Figure 1. Biotransformation of AFB, with putative pathways for CDDO-Im inhibition of carcinogenesis.

populations at high risk for aflatoxin-induced hepatocellular carcinoma. Indeed, Kensler and colleagues have been exploring this avenue for the past decade in clinical trials in Qidong, China, a region of the world with remarkably high incidence of liver cancer due apparently to a combination of endemic hepatitis B viral infections and very high dietary exposure to AFB (2). Using two different, well-established Nrf2 activators, sulforaphane (SFN; an Nrf2 agonist, delivered as the natural glucosinolate derivative, glucoraphanin, from a broccoli sprout tea extract), and the antischistosomal drug, oltipraz, Kensler and colleagues evaluated whether daily treatment for 2 weeks with glucoraphanin or 8 weeks with oltipraz, could reduce the extent of aflatoxin biomarkers, determined through evaluation of AFB–N^7-guanine adducts and aflatoxin-mercapturate in the urine and/or a surrogate biomarker, AFB–albumin adduction, in serum (6–8). On the basis of mechanistic studies in rats, the proposed mechanism of protection conferred by Nrf2 agonists occurs via upregulation of glutathione *S*-transferases (GST) that detoxify the highly reactive epoxide of AFB by conjugation with glutathione (GSH; Fig. 1). They also examined whether treatment with Nrf2 activators increased the elimination of AFB-mercapturate, a breakdown product of the AFB–GSH conjugate. Although there was, not surprisingly, a great deal of intersubject variability, daily treatment with oltipraz increased AFB-mercapturic acid excretion, and treatment with glucoraphanin hinted at a slight decrease in AFB–N^7-guanine adducts in the urine, though the results with glucoraphanin did not reach statistical significance (7, 8).

However, the question remains: can treatment with CDDO-Im be as effective in preventing aflatoxin-induced human liver cancer as it is in rats? Although earlier chemointervention trials suggested some possible effects, other studies are not so encouraging. There is convincing evidence that induction of GST genes, specifically the rat alpha class GST, *rGstA5*, by Nrf2 agonists is the principal, if not sole, mechanism by which Nrf2 agonists decrease AFB–DNA adduct formation and, thus, presumably the cancers that ultimately develop from the DNA damage. Indeed, the remarkable resistance of mice to the hepatocarcinogenic

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effects of AFB is due solely to the constitutive expression *of mGstA3* in the liver. This particular GST has a catalytic efficiency toward AFB-8,9-*exo*-epoxide (AFBO) that is at least 10,000 times greater than any human alpha class GST (9). Surprisingly, both human and non-human primate alpha class GST enzymes display no measurable catalytic activity toward AFBO (10–12). Microsomal and cytosolic enzyme activities using human liver tissue effectively activate AFB to the genotoxic epoxide, but cytosolic fractions of human liver are unable to form a detectable amount of glutathione conjugate (13) under conditions identical to experiments in which mouse and Nrf2-activated rat liver cytosols form extremely high levels of AFB–GSH. Interestingly, one human mu class GST protein, GSTM1-1, does have very low, but measurable, catalytic activity toward AFBO (14, 15), and the GST activity of hepatic cytosolic fraction from the non-human primate *M. fascicularis* was shown to be from mu and not alpha class GSTs (11). Even though the catalytic activity of human GSTM1-1 toward AFBO is several thousand-fold lower than either the mouse (mGstA3-3) or rat (rGstA5-5) forms with high AFBO activity, we demonstrated that even the relatively low catalytic activity of hGSTM1-1 seems to afford significant protection against AFB–DNA adduct formation (16). There is a common polymorphism of the human *GSTM1* gene such that approximately 50% of most populations are homozygous null (i.e., they do not express) for the human *GSTM1* gene, whereas the other approximately 50% have one or two functional alleles. Recent studies in isolated human hepatocytes obtained from *GSTM1*-positive and *GSTM1*-null organ donors demonstrated a 3-fold increase in AFB–DNA adduct formation in the *GSTM1*-null livers (16). Thus, based on assessments of the catalytic activities of human GSTs toward AFBO, it seems unlikely that induction of human alpha class GSTs by Nrf2 agonists would have any significant effect on AFB–DNA adducts, because there is no evidence that any alpha class GST in humans has AFBO-detoxifying capabilities, in stark contrast with rats. Furthermore, *in vitro* studies with SFN in human hepatocytes have failed to demonstrate induction of any human GST, even though other Nrf2-regulated genes such as glutamate cysteine ligase catalytic and modifier subunits (*GCLC* and *GCLM*), NADPH-quinone oxidoreductase 1 (*NQO1*), and thioredoxin reductase 1 (*TXNRD1*) are significantly upregulated by SFN, indicating that the remarkable protective effect against AFB–DNA adducts in human hepatocytes conferred by SFN pretreatment was due to inhibition of AFB activation to the 8,9-epoxide and not through an increase in GST conjugation (17). The inhibition of AFB activation was the result of the extraordinary ability of SFN to block endogenous ligand activation of the pregnane X-receptor that, in primary human hepatocyte cultures, seems to drive most of the expression of CYP3A4 and perhaps CYP1A2 that are necessary for AFB activation (18; Fig. 1). However, treatment of *GSTM1*-positive and *GSTM1*-null human hepatocytes with SFN resulted in the same level of reduction of AFB–DNA adducts, indicating that SFN did not increase detoxification of AFB epoxide (17). Thus, not only do human alpha GSTs lack catalytic activity, neither alpha class *GST* nor *GSTM1* genes seem to be inducible by chemical Nrf2 activators. Consistent with this observation is the apparent lack of any ARE consensus sequences in the 5′ flanking region of human *GSTA1* (19) or, preliminarily, any other human alpha, mu, or pi class *GST* (20), which raises some doubt as to whether the orthologous human genes are inducible via the Nrf2 pathway.

Is it possible that CDDO-Im is providing protection against AFB-induced tumors by inhibiting early-stage carcinogenic processes other than AFB–DNA adduct formation? That is most certainly a possibility. However, Kensler and colleagues (21) have recently shown that pretreatment of *GstA3*-knockout mice—which are highly sensitive to the acute toxicity and genotoxic effects of aflatoxin (22)—with CDDO-Im had no significant effect on AFB–DNA adduct formation in these mice, which argues somewhat against "non–GST"-mediated mechanisms behind the anticarcinogenic effects of CDDO-Im toward AFB-induced liver tumors.

Perhaps the most intriguing results from this study come from the nearly complete protection against both early preneoplastic lesions (GSTP + foci) and later hepatocellular carcinoma, even though there was clearly a great deal of genomic damage through AFB adduction in the CDDO-Im rats. CDDO-Im treatment substantially reduced the amount of AFB–DNA adducts formed, as indicated by both a large increase in the amount of AFB-mercapturate excreted in the urine and a significantly lower amount of AFB–N^7-guanine adducts eliminated in the urine of the CDDO-Im–treated rats relative to untreated rats. Although the treatment clearly induced *rGstA5* gene expression, which significantly enhanced GSH conjugation of the genotoxic AFB–epoxide, the treatment did not completely eliminate AFB-DNA adduct formation, though it did completely eliminate tumor formation and nearly all early lesions. The authors argue that these results provide compelling evidence that an organism must experience relatively large amounts of DNA damage—i.e., a "threshold for carcinogenic response"—before the DNA damage ultimately gives rise to hepatocellular carcinoma(s).

There are a relatively large amount of data on aflatoxins demonstrating an essentially linear relationship between administered dose and the extent of AFB–DNA adducts, as well as an inferred linear relationship between AFB–DNA adducts and eventual tumor development (13, 23). A recent article by Williams (24) reported preliminary evidence of linearity in liver tumor response following AFB treatment even at very low doses. Although the recent findings of Johnson and colleagues certainly demonstrate that there is a strong deviation from linearity between AFB–DNA adduct burden and consequent hepatocellular carcinoma development, the failure of tumors to develop in the CDDO-Im–treated animals may be due in part to CDDO-Im–specific effects on downstream events that interfere with the ultimate progression of early-initiated cells. In addition to its canonical role as a regulator of antioxidant defense, Nrf2 has been shown to interact with myriad signaling pathways

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within the cell, including those controlling cellular growth, proliferation, nutrient sensing (PI3K/AKT/mTOR pathway), and differentiation (Notch1), as well as modulating the expression of genes involved in inflammation, gluconeogenesis, β-oxidation, and lipogenesis (25). Furthermore, CDDO-Im has been shown to block NF-κB (a transcription factor responsible for the production of cytokines and subsequent inflammatory response) signaling through direct inhibition of its cytoplasmic inhibitor IκB kinase (26). Thus, it is possible that if CDDO-Im treatment altered other cellular processes, such as inflammation, that enhance the elimination of initiated cells or otherwise interfere with promotion and progression of initiated cells, the apparent "threshold" for AFB–DNA damage needed to result in hepatocellular carcinoma (i.e., the lack of linearity between AFB–DNA adduct burden and tumor development) as presented here may not apply to very low, chronic exposure to AFB, which could, in fact, be linearly related to tumor development.

Nevertheless, Johnson and colleagues provide the most compelling experimental evidence to date supporting the concept of a threshold for AFB-induced DNA adductions and tumor development. In light of the debate surrounding linear versus threshold approaches to risk assessment for carcinogenesis at low doses, the implications of these findings cannot be understated. Currently, the Environmental Protection Agency's Carcinogen Assessment Guidelines (27) require that the agency use a linearized ("nonthreshold") model when extrapolating relatively high-dose animal tumor responses to predict tumor incidence at much lower dose human exposures for genotoxic carcinogens, unless there are compelling mechanistic data to warrant use of a "nonlinear" ("threshold") approach: "When adequate data on mode of action provide sufficient evidence to support a nonlinear mode of action for the general population and/or any subpopulations of concern, a different approach—a reference dose/reference concentration that assumes that nonlinearity—is used." (27; p. A-9). As noted above, there is a long history of "linearity" between aflatoxin dose and the extent of DNA adduction and from that, an assumption that tumor response would also be linear at low doses. Although the data presented by Johnson and colleagues strongly suggested that AFB–DNA adduct formation and tumor response are not linearly related, no dose–response assessment was done in this study. Given the relatively small sample size and the high dose of AFB received, these data alone cannot eliminate the possibility that there is a proportional relationship between AFB–DNA adducts and tumor response at much lower doses, as suggested by the data from the trout carcinogenesis bioassay reported by Williams (24). It will be interesting to see whether the remarkable protection against tumor development provided by CDDO-Im in AFB-treated rats applies to other mutagenic carcinogens and additionally, whether CDDO-Im affords protection from neoplastic promotion and progression in other models of chemical mutagenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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