Complete Protection against Aflatoxin B₁-induced Liver Cancer with a Triterpenoid: DNA Adduct Dosimetry and Genotoxic Threshold

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Complete Protection against Aflatoxin $B_1$-Induced Liver Cancer with a Triterpenoid: DNA Adduct Dosimetry, Molecular Signature, and Genotoxicity Threshold

Cancer Prevention Research 7:658-665, 2014

Collaborative efforts for nearly 4 decades
General Background of Aflatoxins as Acute Hepatotoxin and Hepatocellular Carcinogen
Aflatoxins

- Discovered in UK ~1960 in moldy, toxic animal feed
  - Lethal protein component to poultry feed (“Turkey X disease”)
- Chemical and biological properties
  - Highly fluorescent, heat stable
  - Multiple forms - aflatoxin B₁ most toxic/carcinogenic
  - Liver carcinogen in virtually all animals tested
  - Acute toxicant and carcinogen for humans
- Frequent contaminant of improperly stored food crops
  - Produced by strains of mold Aspergillus flavus
    (A. flavus toxin = “Aflatoxin”)
  - Spores globally distributed in soil
  - Mold grows on food crops not properly dried
AFB₁ as Hepatocellular Carcinogen

Sensitive

µg/kg/day

Intermediate

? [Human] ?

Resistant

mg/kg/day

Rainbow Trout
Pekin Duck
Fischer Rat
Sprague-Dawley Rat
Zebrafish, Guppy, Medaka

Brook Trout, Tree Shrew
Hamster
Squirrel Monkey
Rhesus, African Green, &
Cynomolgus monkey

Chicken
Mouse
Amphibians

Channel Catfish, Salmon
Hepatic Carcinogenesis: Aflatoxins

- Aflatoxins-induced liver cancer in rats: 1963
- Rainbow trout (*Salmo gairdneri*) sensitive
  - Cheap & easy to use large numbers
- Mouse resistant to aflatoxin
  - Activates AFB₁, but detoxifies efficiently
- Fischer (F344 strain) very sensitive
  - Carcinogenic response: males > females
  - Hepatocellular carcinomas - 1 to 2 years
  - 40 rats/arm: large & expensive experiment
Worldwide Morbidity and Mortality for Liver Cancer

Incidence: 782,000 estimated new cases

- China: 50.5%
- India: 3.5%
- East & Central Asia: 21.2%
- Latin America & the Caribbean: 4.2%
- Oceania: 3.9%
- Europe: 8.1%
- Sub-Saharan Africa: 5.0%
- Middle East & North Africa: 3.4%

Mortality: 746,000 estimated deaths

- China: 51.4%
- India: 3.6%
- East & Central Asia: 20.2%
- Latin America & the Caribbean: 4.2%
- Oceania: 0.3%
- Europe: 8.3%
- Sub-Saharan Africa: 5.0%
- Middle East & North Africa: 3.4%

World Cancer Report 2014, IARC
Worldwide Morbidity and Mortality for Liver Cancer

Incidence: 782,000 estimated new cases

Mortality: 746,000 estimated deaths

World Cancer Report 2014, IARC
NATIONAL CANCER INSTITUTE
10-YEAR MORTALITY TRENDS

MEN

- Liver & IBD: 2.8*
- Soft Tissue Inc. Heart: 1.1*
- Pancreas: 0.3*
- Melanoma: 0.1
- Bladder: 0
- Brain & ONS: -0.3
- Oral Cavity: -0.7*
- Esophagus: -0.8*
- Kidney: -0.5*
- Leukemia: -1.0*
- Myeloma: -1.8*
- Non-Hodgkin Lymphoma: -2.2*
- Larynx: -2.5*
- Lung & Bronchus: -2.7*
- Colon & Rectum: -2.9*
- Stomach: -3.3*
- Prostate: -3.5*

AVERAGE ANNUAL PERCENT CHANGE (AAPC) 2003-2012

WOMEN

- Liver & IBD: 2.2*
- Corpus & Uterus: 1.1*
- Pancreas: 0.4*
- Brain & ONS: -0.3
- Bladder: -0.4*
- Cervix: -0.9*
- Kidney: -1.0*
- Leukemia: -1.2*
- Gallbladder: -1.2*
- Myeloma: -1.2*
- Oral Cavity: -1.3*
- Lung & Bronchus: -1.4*
- Breast: -1.4*
- Ovary: -1.9*
- Stomach: -2.0*
- Colon & Rectum: -2.6*
- Non-Hodgkin Lymphoma: -2.9*

* AAPC is significantly different from zero (p<.05).

Source: Annual Report to the Nation on the Status of Cancer 1975-2012
Incidence of Hepatocellular Carcinoma in USA

Highly Reproducible and Quantitative Animal Model for Aflatoxin B$_1$-induced Hepatocellular Carcinogenesis
Aflatoxin forms several different DNA adducts

Aflatoxin forms several different DNA adducts. AFB1-N7-Gua and AFB1-FAPY are shown in the diagram. Which adducts are mutagenic?
Search for the Most Mutagenic Lesion

Aflatoxin B1 (AFB1) → AFB1-8,9-Oxide → FAPY-Major ↔ Other FAPY Rotamers → AFB1-N7-Gua → AP Site

Smela et al., 2002
Sequential steps

1. Initiation
   - Rapid, DNA damage, mutations

2. Post-initiation events (promotion)
   - Over relatively long time

3. Progression
   - Late event
Quantitative Predictive Rat Model

- **Rat:** F344 or Sprague-Dawley
  - 100 to 110 g at initial dose of AFB$_1$
  - Physiologically cannot vomit

- **Caging:** suspended wire mesh

- **Diet:** AIN 76A or AIN 93 series
  - Dietary acclimation

- **Foci analysis:** weeks post-initiation
  - Initially, 16 weeks
  - **Currently, 5 weeks**
Quantitative Model (cont.)

- Putative preneoplastic lesions – enzymatic positive foci
  - gamma glutamyl transpeptidase positive
  - adenosine triphosphatase deficiency
  - glucose-6-phosphatase deficiency
- $^3$H-thymididine labeling
- Glutathione S-transferase (GST-P foci)
  - A fetal enzyme liver and placental form
  - Immunohistochemical assay
  - Acetone fixed tissue & paraffin imbedded
AFB$_1$ Carcinogenesis in Young Rat

$\downarrow = 25 \mu g$ AFB$_1$

Autopsy

FOCI CANCERS

1 2 3 4 8 9 52 104

Time (weeks)
Weeks following initiation

Fixed liver in paraffin

H & E stain

GST-P stained foci
Microscopic view of two-dimensional section of liver

Morphometric Transformation

• Mean Focal Diameter
• Number Foci/ cm³
• Focal Volume %
Quantitative microscopy-morphometry

**Observed (2-D)**
- Tissue area
- Number of transected foci
- Area of transected focus
- Focal classes

**Calculated (3-D)**
- Foci / unit volume
- Foci / liver
- Mean focal diameter
- Volume % of liver occupied by foci (tumor burden)
Two examples showing general utility of quantitative morphometric analysis of AFB$_1$-induced foci for understanding chemical carcinogenesis and chemoprevention
Schistosoma mansoni
AFB₁ Carcinogenesis in Young Rats

25 μg AFB₁

Time (weeks)

1 2 3 4 8 9 52 104
Inhibition of Hepatocellular Carcinogenesis with 10 doses of AFB₁

<table>
<thead>
<tr>
<th></th>
<th>Focal Incidence</th>
<th>Focal Volume % (mean ± SE)</th>
<th>HCC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aflatoxin</td>
<td>10/10</td>
<td>36 ± 17</td>
<td>5/45 (11)</td>
</tr>
<tr>
<td>Aflatoxin plus oltipraz</td>
<td>4/10</td>
<td>1 ± 1</td>
<td>0</td>
</tr>
</tbody>
</table>

Roebuck et al., 1991
Oltipraz Reduces Aflatoxin-DNA Adduct Formation in Rat Liver
Aflatoxin M₁ (urine) → Aflatoxin M₁ → CYP1A2

Aflatoxin B₁ → Aflatoxin-8,9-epoxide → CYPs 1A2, 3A4

Aflatoxin-8,9-epoxide → GSTs → DNA → AP site → Aflatoxin - N⁷-guanine (urine)

Aflatoxin - mercapturic acid (urine)

Aflatoxin albumin adduct (serum)

other metabolites
Oltipraz intervention: foci, adenomas and adenocarcinomas

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of rats</th>
<th>No lesions</th>
<th>Foci</th>
<th>Adenomas</th>
<th>HCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>No intervention</td>
<td>41</td>
<td>1 (2.4)</td>
<td>4 (9.8)</td>
<td>2 (4.9)</td>
<td>34 (82.9)</td>
</tr>
<tr>
<td>Intervention</td>
<td>40</td>
<td>4 (10)</td>
<td>13 (32.5)</td>
<td>4 (10)</td>
<td>19 (47.5)*</td>
</tr>
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*P<0.05, Fisher’s Exact Test
## Oltipraz intervention: foci, adenomas and adenocarcinomas

### Incidence (%)

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<td>19 (47.5)*</td>
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*P<0.05, Fisher’s Exact Test

- Significant decrease in HCC
- Shift toward less advanced lesions
Chemoprevention by Reduction and Delay
Effect of Oltipraz on Aflatoxin-induced HCC in Rats

Kensler et al., CEBP. 6:603-610, 1997
Reduction in Hepatic AFB-DNA Adduct Levels UNDERESTIMATES Antitumorigenic Efficacy of Oltipraz

Kensler et al, CRT, 1999
Reduction in Hepatic AFB-DNA Adduct Levels UNDERESTIMATES Antitumorigenic Efficacy of Oltipraz

Kensler et al, CRT, 1999
Triterpenoids

- Synthetic analogs of oleanolic acid with anti-inflammatory and antitumorigenic activity
- Inhibit growth, induce cell cycle arrest, and induce apoptosis in breast cancer cell lines
- Inhibit tumor growth in melanoma and leukemia mouse models
- Potent inducers of phase 2 enzymes in vitro
- Functions in part through Nrf2 signaling in vitro

CDDO-I midazolide (TP235)

1Lapillonne et al. Cancer Res 2003
2Place et al. Clin Cancer Res 2003
3Dinkova-Kostova et al. PNAS 2005
4Liby et al. Cancer Res 2005
Protocol for Evaluation of CDDO-Im as an Inhibitor of Aflatoxin-Induced Tumorigenesis in Male F344 Rats

* 1, 3, 10, 30, or 100 µmol CDDO-Im/ kg body weight by gavage

↓ 25 µg aflatoxin B₁ per rat by gavage 6 h after CDDO-Im
CDDO-Im Inhibits Aflatoxin-DNA Adduct Formation in Rat Liver

CDDO-Im (μmol/kg body weight)

Aflatoxin-N7-guanine (pmol/mg DNA)

<table>
<thead>
<tr>
<th>CDDO-Im (μmol/kg body weight)</th>
<th>0</th>
<th>1</th>
<th>3</th>
<th>10</th>
<th>30</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

** P < 0.001
* P < 0.02

\( \bar{x} \pm SE \) (n=4)
CDDO-Im Inhibits Aflatoxin-Induced Tumorigenesis in Rat Liver

Yates et al, Cancer Res, 2006
Paper for Today’s Webinar


Complete Protection against Aflatoxin B$_1$-Induced Liver Cancer with a Triterpenoid: DNA Adduct Dosimetry, Molecular Signature, and Genotoxicity Threshold

Cancer Prevention Research 7:658-665, 2014
Protocol for Induction of HCC with AFB₁ in Rats

Weeks of Age

Bi-weekly

N=43

Lifetime

Sacrifice (N=6/timepoint)

CDDO-Im (30µmol/kg)

AFB₁ (200µg/kg)
Complete Protection Against Aflatoxin-Hepatocarcinogenesis In a Lifetime Bioassay by the Nrf2 Inducer CDDO-Im in Rats

Johnson et al. CAPR (2014)
## Comparison of foci: AFB$_1$ vs AFB$_1$+CDDO-Im

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Age at death (weeks)</th>
<th>Foci number</th>
<th>Observed Mean Diameter, mm (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFB$_1$</td>
<td>35</td>
<td>5</td>
<td>1.30 (1.92-0.85)</td>
</tr>
<tr>
<td>AFB$_1$ + CDDO-Im</td>
<td>93</td>
<td>9</td>
<td>0.43 (0.93-0.15)</td>
</tr>
<tr>
<td></td>
<td>95</td>
<td>2</td>
<td>1.12 (1.82-0.42)</td>
</tr>
<tr>
<td></td>
<td>111</td>
<td>1</td>
<td>0.32</td>
</tr>
</tbody>
</table>
Protocol for Induction of HCC with AFB1 in Rats

Urine: U U U U U U U U

AFB$_1$: * * * * * * * * * * * * *

CDDO-im: 5 6 7 8 9 10 Bi-weekly

Weeks of Age

Sacrifice (N=6/timepoint)

Lifetime N=43
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Days of AFB&lt;sub&gt;1&lt;/sub&gt;</th>
<th>Number of rats</th>
<th>Focal volume % Mean (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>AFB&lt;sub&gt;1&lt;/sub&gt;</td>
<td>8</td>
<td>3</td>
<td>0.01 (0 - 0.04)</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>3</td>
<td>0.25 (0.10 - 0.34)</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>3</td>
<td>3.22 (1.89 - 5.86)</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>6</td>
<td>13.81 (5.25 - 23.11)</td>
</tr>
<tr>
<td>AFB&lt;sub&gt;1&lt;/sub&gt; + CDDO-Im</td>
<td>8</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>3</td>
<td>0.02 (0 - 0.064)</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>4</td>
<td>0.01 (0 - 0.02)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Evaluated at day 28
There are lots of aflatoxin-DNA adducts in rats that never develop liver cancer!

THERE MUST BE A GENOTOXICITY THRESHOLD

**URINE**

### Chart:
- **X-axis:** Days of AFB$_1$ Treatment
- **Y-axis:** Aflatoxin-N7-guanine Excretion (pmol/mg creatinine)
- **Legend:**
  - AFB$_1$
  - AFB$_1$ + CDDO-Im

- Average Dose of AFB$_1$ (µg/day)

- Significant differences indicated with asterisks (*)
There are lots of aflatoxin-DNA adducts in rats that never develop liver cancer!

**THERE MUST BE A GENOTOXICITY THRESHOLD**

![Graph showing Hepatic Aflatoxin-N7-guanine and Hepatic Aflatoxin-FAPyr concentrations over days of AFB₁ treatment.](image)

**LIVER**
CONCLUSIONS

• DNA adduct formation presumed NECESSARY for carcinogenesis by “genotoxic” carcinogens (e.g., aflatoxin)

• Although DNA adducts may lead to mutations, an adduct is not equivalent to a mutation (and not all mutagens are carcinogens)

• Substantial aflatoxin-DNA damage is NOT SUFFICIENT for development of liver cancer

• Carcinogenesis is a multi-step, chronic process and adduct burden likely only accounts for a fraction of disease risk prediction

• Prevention of tumor development can occur with achievable reduction in DNA adduct levels.

• While a carcinogen-DNA adduct (genotoxic) threshold is demonstrable, and biologically plausible, it may not be actionable.