Hormesis in Carcinogenesis: Evidence for Threshold in Carcinogenicity of Non-genotoxic Environmental Carcinogens

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Japan
Environmental carcinogens

- genotoxic or non-genotoxic
- natural or synthetic
- cooking process, contamination, or synthesis in the body
- avoidable or unavoidable
- human intake, 1.5 g/day (B. Ames)
Basic concept in cancer risk assessment

It is generally considered that genotoxic carcinogens have no threshold in carcinogenic potential. This hypothesis has led to acceptance of linear curve that approach zero at low doses for risk assessment. On the other hand, it has been accepted that non-genotoxic carcinogens have threshold. There are, however, limited date available for these hypothesis. Therefore, it is important to resolve this question from the view point of cancer risk assessment and management.
Low-dose carcinogenicity curve: Extrapolation from high to low doses

Genotoxic carcinogenicity

[Diagram showing dose vs. human exposure level with a question mark indicating uncertainty]

Genotoxic carcinogens: mutagenic
act through interaction with DNA
→ irreversible change
unclear carcinogenicity at low doses

Non-genotoxic carcinogenicity

[Diagram showing dose vs. human exposure level with a question mark indicating uncertainty]

Non-genotoxic carcinogens: non-mutagenic
no interaction with DNA
→ reversible change
Threshold in carcinogenicity

A natural question is whether a threshold exists for observed effects of carcinogens. Recently the concepts of “practical” and “perfect” thresholds for genotoxic and non-genotoxic carcinogens have been proposed. In these cases, the carcinogens are associated with a no-observed effect level (NOEL). To answer this question, we examined low dose carcinogenicity of the carcinogens using medium-term bioassay for carcinogens.
Merit of a medium-term bioassay for carcinogens

Normal tissue → Preneolastic lesion → Benign tumor → Cancer

Liver medium-term bioassay → Carcinogenicity test

Liver

Number-Area / unit of glutathione S-transferase placental form (GST-P) positive foci

Incidence of tumors
Low-dose hepatocarcinogenicity of environmental carcinogens

1. Non-genotoxic carcinogens
   - Phenobarbital (PB)
   - $\alpha$-Isomer of benzene hexachloride ($\alpha$-BHC)
   - 1,1-Bis($p$-chlorophenyl)- 2,2,2-trichloroethane (DDT)
   - Ethanol: as a promoter

2. Genotoxic carcinogens
   - 2-Amino-3,8-dimethylimidazo[4,5-$f$]quinoxaline (MeIQx)
   - Diethylnitrosamine (DEN)
   - Dimethylnitrosamine (DMN)
Non-genotoxic carcinogenicity
Most of chemicals involved are non-genotoxic chemicals, acting as P-450 inducers at high doses and exhibiting promoting effects on hepatocarcinogenesis, and the existence of threshold was postulated for the substances acting via epigenetic mechanism.
Phenobarbital (PB)

- Drug, sedative and anticonvulsant
- Mutagenicity: negative
- Hepatocarcinogen
Effect of phenobarbital (PB) at different doses on rat hepatocarcinogenesis (Ito test)

Animals: 180 male F344 rats, 6-week-old

- Diethylnitrosamine (DEN, 200mg/kg, i.p.)
- Saline, i.p.
- PB: 1, 2, 4, 7.5, 15, 30, 60, 125, 250 and 500 ppm (dose in diet)
- Control diet
- 2/3 partial hepatectomy

Endpoint: Liver GST-P positive foci
Development of GST-P positive foci in the liver of rats induced by PB treatment

Number (No./cm²)

<table>
<thead>
<tr>
<th>PB dose (ppm in diet)</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>7.5</th>
<th>15</th>
<th>30</th>
<th>60</th>
<th>125</th>
<th>250</th>
<th>500</th>
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<tbody>
<tr>
<td>* P&lt;0.05</td>
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<td>*</td>
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<tr>
<td>** P&lt;0.01</td>
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Area (mm²/cm²)

<table>
<thead>
<tr>
<th>PB dose (ppm in diet)</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>7.5</th>
<th>15</th>
<th>30</th>
<th>60</th>
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Hepatocarcinogenicity of PB in the rat liver: GST-P positive foci and tumor developments (DEN→PB)

Male F344 rats 6 weeks old

Number (No./cm²) GST-P positive foci (Number/rat) Tumor multiplicity

<table>
<thead>
<tr>
<th>PB in diet</th>
<th>0 3 13 36 wks</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEN (100mg/kg bw,i.p)</td>
<td>-</td>
</tr>
</tbody>
</table>

Number (No./cm²)

* P<0.05
** P<0.01

Tumor multiplicity

* P<0.05
8-OHdG and P-450 in rat liver treated with PB at 500 ppm for 8 days

8-OHdG and CYP3A2

8-OHdG and CYP2B1

control
P-450 total contents and OH radicals generation in the rat liver induced by DEN→PB treatment

8-OHdG and OGG1 mRNA expression levels induced in the rat liver by DEN→PB administration
PCNA positive index within and surrounding area of GST-P positive foci in rat liver

* P<0.05 v.s. DEN

Immunohistochemistry

PCNA (blue) and GST-P (red) double staining
Apoptotic index within and surrounding area of GST-P positive foci in rat liver

Apoptosis (blue) and GST-P (red) double staining

8-OHdG
**Differentially expressed genes in the rat liver after DEN → PB treatment detected by cDNA microarray analysis**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Gene</th>
<th>Alteration</th>
</tr>
</thead>
<tbody>
<tr>
<td>aDEN</td>
<td>Glutathione-S-transferase</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>Cytochrome P450 3A</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>G protein α subunit</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td>Glutamic acid decarboxylase</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td>GABA receptor beta subunit</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td>Glutamic acid decarboxylase (GAD65)</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>Chloride channel ClC-7</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>Phospholipase C</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>MAP kinase p38</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td>Ca-CAM dependent PK</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td>Cytochrome P450 3A</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>Nuclear tyrosine phosphatase</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>Glutathione S-transferase</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>Cytochrome P450 3A, 2C, 4A</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>DOPA decarboxylase</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>Nuclear tyrosine phosphatase</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>NADPH-P450 reductase</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>G protein α subunit</td>
<td>↑</td>
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<tr>
<td></td>
<td>VEGFR 1</td>
<td>↑</td>
</tr>
</tbody>
</table>

*a* VS. non-treated control    *b* VS. DEN
Summary of PB study

1. PB at low doses inhibited the development of GST-P positive foci and tumor formation in the rat liver, whereas PB at high doses exhibited promoting effect of hepatocarcinogenesis (J-shape curve).

2. Inhibition of DNA 8-OHdG formation, decrease of cell proliferation within the GST-P positive foci and induction of apoptosis in the surrounding liver tissue were found in the low dose group.

3. This hormetic phenomenon of PB carcinogenicity supports the concept discussed recently by E. Calabrese and E. A. Baldwin.
\(\alpha\)-Isomer of benzene hexachloride (\(\alpha\)-BHC)

- Agricultural chemical
- A major organochlorine byproduct in the manufacture of lindane
- Mutagenicity: negative
- Hepatocarcinogen
Development of GST-P positive foci in the liver of rats induced by $\alpha$-isomer of benzene hexachloride ($\alpha$-BHC) (Ito test)

Animals: 180 male F344 rats, 6-week-old

- Diethylnitrosamine (DEN, 200mg/kg, i.p.);
- 2/3 partial hepatectomy

Number (/cm²)  Area (mm²/cm²)

<table>
<thead>
<tr>
<th>$\alpha$-BHC dose (ppm in diet)</th>
<th>Number</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.010</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td></td>
<td></td>
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<tr>
<td>0.5</td>
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<td></td>
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<tr>
<td>1</td>
<td></td>
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<td>2</td>
<td></td>
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<tr>
<td>4</td>
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<tr>
<td>7.5</td>
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<td>125</td>
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<td>500</td>
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</tr>
<tr>
<td>1250</td>
<td></td>
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<tr>
<td>5000</td>
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</tbody>
</table>

Statistical significance:

- ** P<0.01
- *** P<0.001
Male F344 rat, 6 weeks old

DEN (100mg/kg bw, ip) followed by α-BHC, 0-500 ppm in diet

GST-P positive foci in the rat liver initiated by DEN followed by α-BHC

* P < 0.05 when compared with DEN alone group
**Effect of α-BHC on oxidative stress**

8-OHdG formation in the rat livers

<table>
<thead>
<tr>
<th>α-BHC dose (ppm)</th>
<th>0</th>
<th>0.01</th>
<th>0.05</th>
<th>0.1</th>
<th>1</th>
<th>50</th>
<th>500</th>
</tr>
</thead>
<tbody>
<tr>
<td>8OHdG/10e5dG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.30</td>
<td>0.31</td>
<td>0.32</td>
<td>0.33</td>
<td>0.34</td>
<td>0.35</td>
<td>0.36</td>
</tr>
</tbody>
</table>

* P<0.05 when compared with DEN alone group

OGG1 mRNA expression in the rat livers

<table>
<thead>
<tr>
<th>α-BHC dose (ppm)</th>
<th>0</th>
<th>0.01</th>
<th>0.05</th>
<th>0.1</th>
<th>1</th>
<th>50</th>
<th>500</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ratio OGG1/β-actin expression</td>
<td>0.80</td>
<td>0.81</td>
<td>0.82</td>
<td>0.83</td>
<td>0.84</td>
<td>0.85</td>
<td>0.86</td>
</tr>
</tbody>
</table>

* P<0.05 when compared with DEN alone group
Effect of $\alpha$-BHC on cell proliferation

PCNA in GST-P positive foci and their surrounding area

TGF-$\beta$ mRNA expression

* $P<0.05$ when compared with DEN alone group

Double immunostaining of GST-P and PCNA
Correlation of some biological markers in $\alpha$-BHC treated rats
Summary of $\alpha$-BHC study

1. Using GST-P positive foci as the endpoint marker, $\alpha$-BHC showed hormetic phenomenon in DEN-initiated hepatocarcinogenesis. (J-shape curve).

2. The possible mechanism of hormesis might involve alterations in xenobiotic metabolism, cytochrome P450 oxidoreductase system, that produce free radicals followed by oxidative stress and consequently pathological change in the liver.
1,1-Bis(p-chlorophenyl)- 2,2,2-trichloroethane (DDT)

- Pesticide
- Mutagenicity: negative
- Hepatocarcinogen
Low dose carcinogenicity of DDT in rat liver
(Male F344, 21-day-old, 16 wks study)

**P<0.01**
Hepatocarcinogenicity of DDT in the rat liver

Male F344 rats
6 weeks old

DEN (100 mg/kg bw. i.p.)

DDT in diet

Tumor incidence

<table>
<thead>
<tr>
<th>ppm</th>
<th>0</th>
<th>0.005</th>
<th>0.5</th>
<th>500</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>77</td>
<td>81</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Tumor multiplicity

<table>
<thead>
<tr>
<th>ppm</th>
<th>0</th>
<th>0.005</th>
<th>0.5</th>
<th>500</th>
</tr>
</thead>
<tbody>
<tr>
<td>No./rat</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>

**P<0.01

**
P-450 contents in the rat liver induced by DEN→DDT

8-OHdG formation and OGG1 mRNA expression in the rat liver induced by DEN→DDT
Alteration of gap junctional intercellular communication (Cx32) and its regulator gene (HNF-1α) in the liver

**P<0.01
Summary of DDT study

1. Inductions of GST-P positive foci and tumors tended to be inhibited by DDT at low dose, whereas DDT at high doses increased GST-P positive foci (J-shape curve, hormesis).

2. OGG1, connexin 32 and HNF-1α expressions showed inverted U-shape curve.
Proposal of a flow scheme toward dose-effect relations, risk assessment and mechanisms of hormesis of non-genotoxic chemical carcinogens

Non-genotoxic hepatocarcinogen

**Low dose**
- Inhibition of oxidative stress (CYP3A2, 8-OHdG)
- Increase of DNA repair
- Inhibition of cell proliferation in the areas of GST-P positive foci
- Suppression of apoptosis in normal-appearing liver cell area
- Protection of GJIC
- Activation of liver detoxification system (CYP2C11, P-450 NADPH oxidoreductase)
- Cell signaling (GABA↑, MAP kinases signaling pathways↑)
- Inhibition of carcinogenesis

**High dose**
- Induction of oxidative stress (P-450, ROS, 8-OHdG)
- Induction of DNA repair
- Increase of cell proliferation in the areas of GST-P positive foci
- Induction of apoptosis in normal-appearing liver cell area
- Inhibition of GJIC
- Inhibition of CYP2C11
- Promotion of carcinogenesis

Perfect threshold in carcinogenicity
Dose-dependence of promotion by ethanol on rat hepatocarcinogenesis
Dose-dependent liver tumor induction by ethanol
Liver cell proliferation with dose-dependence: PCNA indices

* P<0.05
Increase of CYP2E1 expression by ethanol

* P<0.05

Concentration of ethanol

Control

20% ethanol

Protein expression

Increase of CYP2E1 expression by ethanol
Summary of ethanol study

1. Ethanol dose-dependently promoted hepatocarcinogenesis induced by MeIQx, but with no adverse influence at doses of 1% and less, comparable to sensible drinking levels in human.

2. Cell proliferation and CYP2E1 influenced promotion activities of ethanol, without evidence of increase in 8-OHdG, a oxidative DNA damage marker.

3. Hormesis phenomenon was not observed in ethanol-mediated promotion of hepatocarcinogenesis in rats.
Conclusions

1. Hepatocarcinogenicity of non-genotoxic carcinogens, such as PB, α-BHC and DDT showed a hormetic phenomenon (J-shape curve).

2. Alteration to cell proliferation, oxidative damage at high and low doses may have important roles in the hormesis.

3. These non-genotoxic carcinogens have a perfect threshold for their carcinogenicity.
Genotoxic carcinogenicity
2-Amino-3,8-dimethylimidazo[4,5-f]quinoxaline:

- One of heterocyclic amines
- Exists in well-cooked fish and meat
- Mutagenicity: positive
- Hepatocarcinogen
- Human exposure level: 0.2-2.6 µg / day
Induction of rat liver cancers treated with MeIQx for 56 weeks

MeIQx dose (ppm in diet)

**P<0.01**

MeIQx: 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline

(Wakabayashi et al, 1995)
Rat hepatocarcinogenicity of MelQx at low doses

Animals: 1,180 male F344 rats, 21-day-old

No. /cm²

GST-P positive foci

** P<0.01

MelQx dose (ppm in diet)

0 0.001 0.01 0.1 1 10 100

10

100
Formation of MelQx-DNA adducts and 8-OHdG in the rat liver treated with MelQx for 4 weeks

Adduct /10⁷ ntd

8-OHdG /10⁵ dG

DNA adduct

8-OHdG

* P<0.05

** P<0.01
MelQx

Big Blue rat

LacI gene: 30 - 40 copies on chromosome 4 in the F344 rat

Mutation in the lacI gene

lacI lacO αlacZ

Repressor X-gal Blue

Transcriptional

β-galactosidase

In vivo mutagenicity test in Big Blue rats

(Plaque Color Screening Assay)

Infection E.coli

Incubation containing X-gal

Blue plaque=Mutation (+)

White plaque=Mutation (-)

DNA isolation

Liver

Phage

in vitro packaging

Blue plaque=Mutation (+)

White plaque=Mutation (-)
Frequency of LacI gene mutations and development of GST-P positive foci in the liver of Big Blue rats treated with MeIQx for 16 weeks

lacI gene: 30 - 40 copies on chromosome 4 in the F344 rat
Initiation activity of MeIQx at low doses in the rat liver

Animals: 850 male F344 rats, 21-day-old

MeIQx; 0, 0.001, 0.01, 0.1, 1, 10, 100 ppm in diet

Number (No./cm²)

**P<0.01
Risk of liver cancer: Reaction curves for the carcinogenicity markers dependent on the dose of MeIQx

**Control level**

- MeIQx-DNA adduct
- H-ras mutation
- 8-OHdG
- GST-P positive foci
- LacI mutation
- Initiation activity
- Liver cancer

**Response**

- MeIQx doses
Rat hepatocarcinogenicity of \(N\)-nitroso compounds: Induction of GST-P positive foci

**Diethylnitrosamine (DEN)**
- male F344, 21-day-old, 1,957 rats
- 0-16 wks
- DMN (ppm in drinking water):
  - 0.01
  - 0.1
  - 1

**Dimethylnitrosamine (DMN)**
- male F344, 21-day-old, 1,520 rats
- 0-16 wks
- DEN (ppm in drinking water):
  - 0.0001
  - 0.001
  - 0.01
  - 0.1
  - 1

**P<0.01**

**P<0.01**
Conclusion

1. The carcinogenicity markers showed no-effect levels for their response.

2. The genotoxic carcinogens such as MeIQx and DEN have practical threshold for their carcinogenicity.

3. Further studies are required for hormetic effect of genotoxic carcinogens.
Our data demonstrate that some of non-genotoxic carcinogens have hormesis for their carcinogenicity, showing existence of perfect threshold. Genotoxic carcinogens exhibit threshold, at least practical threshold. These conclusions may introduce new concept for cancer risk assessment and management.
Collaborators

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Tsuda, Hiroyuki (Experimental Pathology and Chemotherapy Div., National Cancer Center Research Institute; at present, Nagoya City University Med. Sch.)

Wakabayashi, Keiji (Cancer Prevention Research, National Cancer Center Research Institute)
Detection of H-ras mutation: Thermosequenase cycle end labeling (TCEL) method

PCR

Electrophoresis Scanning

Primer labeling

tissue → DNA → H-ras

Annealing

Addition

Primers

Taq DNA polymerase

Temp. change

Wild type

Mutant type

[a-33P]ddGTP

[a-33P]ddGTP

Primer labeling → Electrophoresis → Scanning
Frequencies of H-ras mutation and GST-P positive foci in the liver of rats treated with MeIQx

Detection of H-ras mutation: Thermosequenase cycle end labeling (TCEL) method
**LacI** Mutation frequency and development of GST-P positive foci in the liver of Big Blue rats treated with DEN or 2-acetaminofluorene (2-AAF) for 16 weeks

**Graph 1:**
- **x-axis:** DEN (ppm in drinking water)
- **y-axis:** MF (No./10^6)
- **y-axis:** GST-P (No./cm²)
- **Legend:**
  - △ LacI mutations
  - ● GST-P positive foci

**Graph 2:**
- **x-axis:** DEN (ppm in drinking water)
- **y-axis:** MF (No./10^6 phages)
- **Legend:**
  - □ AT→TA Transversion
  - ■ AT→GC Transition

**Graph 3:**
- **x-axis:** 2-AAF (ppm in diet)
- **y-axis:** MF (No./10^6)
- **y-axis:** GST-P (No./cm²)
- **Legend:**
  - △ LacI mutations
  - ● GST-P positive foci

*P < 0.05
**P < 0.001
Etiology of human cancer  (R. Doll 1981)
Potential mechanisms mediating hormesis in carcinogenesis

DNA repair (Ogg1), Cx32, HNF-1α
Detoxification enzymes activities (CYP21C11, OR)

GST-P positive foci and tumors
Oxidative DNA damage, 8-OHdG
Proliferation in GST-P positive foci
Apoptosis in surrounding area
Chemical carcinogenesis mechanisms

- Carcinogen
  - Inactivation
  - Metabolic activation: ultimate carcinogen
    - Non-DNA
      - DNA repair
        - DNA repair error
          - DNA: epimorphosis
            - Cancer-irrelative mutations
            - Mutation: irreversible change
              - Promotion: progression
                - Preneoplasia: cell proliferation
                  - Apoptosis
                    - Cancer: malignancy

- Initiation
  - Apoptosis

- Preneoplasia: cell proliferation
  - Apoptosis
    - Cancer: malignancy