defines the relationship between the dose of an agent and the probability of induction of a carcinogenic effect, and is one of the most important components of carcinogen risk assessment. The dose–response relationship for genotoxic carcinogens is generally assumed to be linear without a threshold dose below which carcinogenic effects are absent, meaning that genotoxic carcinogens may pose some risk at any level of exposure [1–3], although there is no definitive experimental evidence to support this suggestion.

2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), a heterocyclic amine detected in the cooked meat and fish, is a potent genotoxic carcinogen [4,5]. MeIQx induced hepatocellular carcinoma in male F344 rats at high doses [6,7]. However, our recent low-dose studies showed the existence of no-observed effect levels (NOEL) for MeIQx on induction of glutathione S-transferase placental form (GST-P)-positive foci in the livers of F344 rats [8,9]. GST-P-positive foci is a well-established preneoplastic lesion in the liver of rat and has been accepted as a marker for assessing carcinogenic potential in the liver [10]. Moreover, GST-P-positive foci in the liver has been suggested to be a useful end-point marker in assessment of carcinogenic effects of environmentally relevant concentrations of liver carcinogens [11]. More recently, we found that low doses of MeIQx did not increase mutation frequencies in rat livers and the dose–response curve for mutation frequency is nonlinear in an in vivo mutagenicity assay using male Big Blue rats with genetic background of F344 [12]. These findings argue against the linear no-threshold risk assessment model for genotoxic carcinogens and indicate that there is a practical threshold for hepatocarcinogenetic effects of MeIQx

2. Materials and methods

2.1. Animals

One-hundred and eighty male BN rats and 180 male F344 rats, 20-days old, were obtained from Charles River Japan, Inc. (Atsugi, Kanagawa, Japan). The animals were housed in polycarbonate cages (5/cage) in a room with a targeted temperature of 23 ± 2 °C, humidity of 55 ± 5%, and a 12 h light/dark cycle and ad libitum access to food and tap water.

2.2. Chemical and diets

MeIQx (purity, 99.9%) was purchased from Nard Institute, Nishinomiya, Japan. Basal diet (powdered MF) and MeIQx diets were prepared at Oriental Yeast Co. Tokyo, Japan, and concentrations of MeIQx in the diets were confirmed by HPLC. The lowest level of MeIQx fed in the diet was 0.1 ppm based on previous experiments showing this dose had no effect on GST-P-positive foci development in F344 rats [8].

2.3. Experimental procedures

At 21 days of age, BN and F344 rats were randomized into groups of 30 rats each. As shown in Table 1, BN rats (groups 1–6) and Fisher rats (groups 7–12) were fed diets containing 0, 0.1, 1, 5, 10, or 100 ppm of MeIQx. Body weight, water and food consumption were measured weekly. All rats were sacrificed under ester anesthesia after 16 weeks of treatment for examination of GST-P-positive foci in the liver. Briefly, livers were excised, weighed and 3 slices each from the left lateral, medial, and right lateral lobes were fixed in 10% phosphate-

Table 1
Data on body and liver weights in BN and F344 rats

Group	MeIQx (ppm)	No. of rat	Body weight (g)		Liver weight	
			Initial	Final	Absolute (g)	Relative (%)
BN rat						
1	0	30	37±3	333±21	7.8±1.0	2.3±0.3
2	0.1	30	37±4	335±23	7.6±1.3	2.3±0.3
3	1	30	37±3	337±21	7.6±1.1	2.3±0.3
4	5	30	37±3	341±18	8.0±1.1	2.4±0.3
5	10	30	37±3	339±16	8.2±1.2	2.4±0.3
6	100	30	37±4	321±18	8.2±1.1	2.5±0.3
F344 rat						
7	0	30	28±3	303±19	8.0±1.5	2.7±0.5
8	0.1	30	28±3	302±18	7.9±1.4	2.6±0.4
9	1	30	28±3	297±16	7.8±1.4	2.6±0.4
10	5	30	28±3	302±17	8.0±1.3	2.6±0.3
11	10	30	28±3	297±19	7.8±1.1	2.6±0.4
12	100	30	28±3	292±12[a]	9.1±1.2[a]	3.1±0.3[a]

[a] Significantly different from group 7.

2.4. Statistical analysis

All mean values are reported as the mean±SD. Differences between mean values of control and treated groups in F344 or BN rats was analyzed using Dunnett two-tailed post hoc test [8]. Student's *t*-test or Welch's *t*-test were applied for identifying differences between F344 and BN groups. *P* values of less than 0.05 were considered to be statistically significant. All statistical analyses were performed using a StatView-J5.0 program (Abacus Concepts, Inc., Berkeley, CA).

3. Results

3.1. General observations

All the rats survived in good condition until the scheduled sacrifice. No macroscopic lesions were noted in any tissue in any rats. As shown in Table 1, the body weights of BN groups were higher compared to F344 groups prior to the start of the study and continued to be higher throughout the experiment. Final body weights were significantly decreased in F344 rats administered 100 ppm MeIQx compared to controls. The similar decrease in body weight is also observed in the BN group administered 100 ppm MeIQx but it is not statistically significant. Administration of MeIQx had no effects on liver weights in BN rats. Absolute and relative liver weights were significantly increased in F344 rats given 100 ppm MeIQx compared to control. There was no significant difference in food or water intake among groups throughout the experiment (data not shown).

3.2. Induction of GT-P-positive foci in the liver

Data for GST-P-positive foci are given in Table 2. In BN rats, total numbers of GST-P-positive foci per unit area of the livers of groups administered 0.1–5 ppm of MeIQx did not differ from the control value (0 ppm group), in contrast to a tendency to increase observed with 10 ppm and a prominent, statistically significant increase with 100 ppm MeIQx. Number of GST-P-positive foci comprising 2–4 cells, 5–10 cells, or ≥11 cells also significantly increased only in 100 ppm group compared to the control group.

The background level of GST-P-positive foci in F344 rats was lower than in BN rats. Likewise, F344 rats showed lower values for number of GST-P-positive foci than BN rats given same dose of MeIQx. The tendency to increase in number of GST-P-positive foci is similar to that for BN rats. MeIQx up to 5 ppm had no effects on the formation of GST-P-positive foci. In F344 rats given 10 ppm MeIQx a tendency to increase was noted, significant increases in the total number and individual numbers of different size of GST-P-positive foci reached for with 100 ppm MeIQx.

Table 2
Development of GST-P-positive foci in the livers of BN and F344 rats treated with various doses of MeIQx

Group	MeIQx (ppm)	No. of rat	Size distribution of GST-P-positive foci (No./cm^2)			
			2–4 cells	5–10 cells	≥ 11 cells	Total
BN rat						
1	0	30	0.16±0.21	0.06±0.12	0.02±0.10	0.24±0.29
2	0.1	30	0.14±0.23	0.03±0.08	0.03±0.19	0.19±0.30
3	1	30	0.12±0.21	0.04±0.09	0.04±0.14	0.20±0.33
4	5	30	0.23±0.33	0.11±0.19	0.02±0.09	0.36±0.49
5	10	30	1.17±0.98	0.42±0.57	0.06±0.14	1.64±1.43
6	100	30	13.26±7.07[a]	7.37±4.78[a]	4.25±3.88[a]	24.88±14.67[a]
F344 rat						
7	0	30	0.01±0.05[c]	0[c]	0	0.01±0.05[c]
8	0.1	30	0.03±0.08[c]	0	0.01±0.05	0.04±0.10[c]
9	1	30	0.07±0.15	0[c]	0	0.07±0.15
10	5	30	0.08±0.16[c]	0.01±0.05[c]	0	0.08±0.18[c]
11	10	30	0.29±0.49[c]	0.04±0.12[c]	0[c]	0.33±0.59[c]
12	100	30	3.60±2.22[b,c]	1.83±1.33[b,c]	0.99±1.01[b,c]	6.41±4.04[b,c]

[a] Significantly different from group 1.
[b] Significantly different from group 7.
[c] Significantly different from corresponding BN rat group.

4. Discussion

The present study showed that low doses of MeIQx had no effects on induction of liver GST-P-positive foci in BN rats and therefore demonstrated the existence of NOEL for hepatocarcinogenicity of this genotoxic carcinogen. The same NOELs were also observed in F344 rats, although background level of GST-P positive foci is different in the two rat strains. Results for F344 rats are consistent with our previous work in which NOELs of MeIQx were suggested to be less than 10 ppm [8,9]. These findings further support our notion that a practical threshold dose for MeIQx hepatocarcinogenicity exists in rats.

With respective to susceptibility to hepatocarcinogenesis, BN rats have been found to be resistant than F344 rats to hepatocellular carcinoma formation in the resistant hepatocyte (RH) protocol in which rats are initiated with diethylnirtrosamine (DEN), and followed by dietary administration of 2-acetylaminofluorene (2-AFF) and partial hepatectomy [15,16]. In the above model, neither number of GST-P positive lesions nor neoplastic hepatic nodules in BN rats differ from F344 rats, however, both the size of GST-P positive lesions and the size of neoplastic hepatic nodules were significantly higher in F344 rats than in BN rats and were predictive of subsequent development of hepatocellular carcinomas. In contrast, in the present model, both total numbers of GST-P positive foci and the numbers of larger GST-P-positive foci (containing more than 11 hepatocytes) induced by carcinogenic level of MeIQx (100 ppm) are significantly higher in BN rats than in F344 rats. Considering the results of the size of GST-P-positive foci, the possibility could not be excluded that BN rats are more susceptible than F344 rats to hepatocarcinogenicity of MeIQx in the present model. Further studies using liver tumor as endpoint marker will be necessary to compare susceptibilities of BN and F344 rats to hepatocarcinogenicity of MeIQx.

There is increasing evidence that dose–response curve of genotoxic carcinogen is nonlinear and a practical threshold dose exists. In addition to MeIQx, several genotoxic carcinogens have also been shown to have thresholds for their carcinogenic effects including liver carcinogen DEN [8,17] and 2-AAF [18–20] and colon carcinogen 2-amino-1-methyl-6-phenolimidazo[4,5-*b*]pyridine in rats [21]. Analysis of dose response relationship is one of the most important components of carcinogen risk assessment. Data accumulation in this research field would facilitate not only the risk assessment model

development and but also the establishment of guide for practical recommendations for cancer risk assessment.

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Alpha-benzene hexachloride exerts hormesis in preneoplastic lesion formation of rat hepatocarcinogenesis with the possible role for hepatic detoxifying enzymes

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Abstract

Recently there has been a shift in the prevailing paradigm regarding the dose dependence of carcinogen action with increasing acceptance of hormesis phenomenon, although underlying mechanisms remain to be established. To ascertain whether alpha-benzene hexachloride (α-BHC) might act by hormesis, rats were initiated with diethylnitrosamine and then α-BHC ranging from 0.01 to 500 ppm was administered in the diet for 10 weeks. The highest concentration of α-BHC significantly increased the number and area of glutathione *S*-transferase placental form (GST-P) positive foci, preneoplastic lesions in the liver, but its low dose, 0.05 ppm, caused significant reduction, showing a J-shape dose-response curve. The proliferating cell nuclear antigen positive index for GST-P positive foci in the low dose-treated group was significantly reduced. The dose response curves of CYP450 content, NADPH-P450 reductase activity and 8-hydroxydeoxyguanosine formation revealed the same pattern as GST-P positive foci data. The response curves of CYP2B1 and 3A2 in their activities, protein and mRNA expression showed a threshold but CYP2C11 activity exhibited an inverted J-shape. These results might suggest the possibility of hormesis of α-BHC at early stages of rat hepatocarcinogenesis. The possible mechanism involves induction of detoxifying enzymes at low dose, influencing free radical production and oxidative stress, and consequently pathological change in the liver.

Keywords: Alpha benzene hexachloride; Hormesis; Early stage of hepatocarcinogenesis; GST-P positive foci; Cytochrome P450

1. Introduction

α-Benzene hexachloride (α-BHC), a major by-product in the manufacture of lindane (γ-BHC), has been used in admixture with lindane for agriculture

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purposes. It persists in the environment and contaminates the food chain at an average human exposure level of 10 ng/kg bw [1]. Among 8 isomers of BHC, the α-isomer has been categorized as a non-genotoxic carcinogen because it induces hepatocellular carcinomas in rodents with high dose long-term administration but lacks mutagenicity in the Ames test [2]. Concern about the possible adverse health effects of long-term exposure to this compound in humans has led to it being banned or restricted in industrialized countries. Though it has been classified as a non-genotoxic carcinogen, some investigations demonstrated that high concentration of α-BHC exhibited inhibiting effect on liver tumor formation induced by some known carcinogens in rats [3,4]. These results are raise concerns as to how α-BHC plays a role in hepatocarcinogenesis. Our previous study demonstrated that while a high concentration of α-BHC promotes liver carcinogenesis, low doses tend to decrease the formation of rat liver preneoplastic lesions, glutathione *S*-transferase placental form (GST-P)—positive foci, in the medium-term rat liver bioassay (Ito test) [5,6]. From these results one may reason that α-BHC may also exert inhibitory effects on hepatocarcinogenesis at low doses.

Non-genotoxic carcinogens are the chemicals that induce neoplasia without themselves or their metabolites reacting directly with DNA. They may cause oxidative DNA damage and thereby indirectly stimulate hyperplastic or neoplastic responses. Oxidative stress has been defined as an imbalance between oxidants and antioxidants in favor of the former, resulting in an overall increase in cellular level of reactive oxygen species [7]. The correlation between induction of cytochrome P450 isozymes and the subsequent reactive oxidants production warrants consideration as a possible mechanism for the induction of oxidative stress and tumor promotion after exposure to a number of chlorinated and nonchlorinated compounds such as dieldrin, lindane, and phenobarbital [8].

Distinction of dose–response curves between genotoxic and non-genotoxic carcinogens has been made with reference to different biological markers. A dose threshold for tumor induction has been assumed for non-genotoxic carcinogens with no-observed effect levels (NOELs), while genotoxic carcinogens have been assumed to exhibit strict dose dependence even at exceedingly low doses [9–11]. However, some risk assessment studies have pointed also to a threshold in response curves with some genotoxic carcinogens at very low doses [12–14,45]. Numerous toxicological studies have shown hormesis phenomenon [15–17]. This phenomenon, which shows either a decrease below the control level at a low dose followed by an increase at a high dose, is called a U- or J-shape relationship, or vice versa, termed an inverted U-shape or β-shape curve. A novel threshold model of carcinogenesis has been proposed [18,19] and hormesis might occur in response to the disruption of homeostasis [20] but the evidence depends on the experimental design, test chemical concentrations, duration of exposure, end-point parameters and the statistical analysis that is applied [18].

To verify the hormesis effects of α-BHC on the early stages of hepatocarcinogenesis in rats and cast light on its mechanisms, diethylnitrosamine (DEN), a well-known hepatocarcinogen, was chosen as an initiator in the present medium-term carcinogenicity test based on the two-step carcinogenesis theory. GST-P positive foci were used as the end point marker of hepatocarcinogenesis. In addition, cell proliferation, oxidative stress and xenobiotic metabolizing enzymes were chosen as parameters for investigation.

2. Materials and methods

2.1. Chemicals

α-BHC (CAS no 319-84-6, purity 100%) was purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan) and DEN from Tokyo Kasei Kogyo (Tokyo, Japan)

2.2. Animals and experimental protocol

Male 5-week-old F344 rats were purchased from Charles River Japan Inc.(Atsugi Japan) and maintained on MF powdered diet (Oriental Yeast, Co., Tokyo, Japan), containing γ-BHC <0.005 ppm, DDT <0.05 ppm and PCB <0.01 ppm, in an animal room with a 12 h (8:00 20:00) light/dark cycle, at a constant temperature of 25 ± 1 °C and a relative humidity of 55 ± 5%. The animals were observed

daily and were used for the experiment after a week acclimation period.

The rats were divided into 7 groups, 12 rats per group, and all injected with 100 mg/kg bw of DEN via intraperitoneum weekly 3 times. One week after the last injection, α-BHC containing diet at concentration of 0, 0.01, 0.05, 0.1, 1, 50 or 500 ppm was given. After 10 week of tested chemical administration, rats were anesthesized under diethylether. Then, liver was flushed with an ice-cold perfusion buffer (1.15% KCl buffer pH 7.4 containing 1 mM EDTA and 0.25 mM phenylmethylsulfonyl fluoride) until became pale and was immediately dissected. Three to four mm thick sections from the right, medial and left lateral lobes were fixed in 10% buffered formalin and embedded in paraffin for routine histological and immunohistochemical analyses. The remaining liver tissue was frozen under liquid nitrogen and stored at −80 °C until applied for biochemical and molecular analyses.

2.3. Immunohistochemistry for GST-P and double staining of PCNA

After deparaffinization, 3 μm liver sections were immunostained for GST-P as described by Kitano et al. [16] using rabbit anti rat GST-P (MBL Co.Ltd., Nagoya, Japan, 1:1000 dilution, 4 °C, overnight), biotin-labeled goat anti-rabbit IgG and an avidin-biotin complex method (ABC kit; Vector Laboratories, Burlingame, CA). 3′-3′-Diaminobenzidine (DAB) was applied for final color development. The number and area of GST-P positive foci comprising more than two cells and area more than 0.2 mm in cross-section were quantified using an Image Processor for Analytical Pathology (IPAP) system (Sumica Technos, Osaka, Japan). Hepatocyte proliferative activity was determined using the double-staining technique by combination of GST-P immunohistochemistry as above with the enhanced polymer one-step method for proliferating cell nuclear antigen (PCNA) (Dako EPOS, Dako Cytomation, CA) and conjugated with a labeled polymer, alkaline phosphatase (Envision goat anti-rabbit IgG labeled polymer alkaline phosphatase, Dako Cytomation, CA). The color of GST-P positive foci was developed in red by the new fuchsin substrate system (Dako Cytomation, CA) while positive PCNA nuclei stained brown by DAB. PCNA indices were estimated separately for GST-P positive and negative areas as numbers of positive nuclei per 1000 hepatocytes.

2.4. Analysis of hepatic phase I and II enzyme protein levels and activities

Frozen liver homogenates were prepared in ice-cold perfusion buffer using a Teflon-glass homogenizer. Cytosolic and microsomal fractions were prepared from individual rat livers and performed by differential centrifugation. Cytosolic glutathione *S*-transferase (GST) activity was assessed with 1-chloro-2,4-dinitrobenzene serving as a substrate [21]. Microsomal portions were assayed for P450 content [22]. NADPH-P450 reductase activity was measured as described elsewhere [23].

The activities of CYP450 isozymes were determined by hydroxylation of testosterone using HPLC [24]. SDS-PAGE was performed by the method of Laemmli and then western blot analysis was carried out as reported previously [25]. Rabbit anti-rat CYP2B1, 2C11/6, 2E1, 3A1/2 and NADPH-P450 reductase antibodies used were prepared by Prof. Y. Funae and his group [26]. Protein concentrations were measured using the Lowry method.

2.5. Measurement of 8-hydroxydeoxyguanosine (8-OHdG) formation

The level of 8-OHdG formation in rat liver was measured by the method described previously [27]. Briefly, DNA was extracted from liver using a DNA Extractor WB kit (Wako Pure Chemical Industries Ltd, Osaka, Japan), containing NaI, deferoxamine mesylate (Sigma Chemical Co., St Louis, MO) and RNase (Wako Pure Chemical Industries Ltd), then digested to nucleosides by combined treatment with nuclease P1 (Yamasa Shoyu Co. Ltd, Chiba, Japan) and alkaline phosphatase (Sigma Chemical Co., St Louis, MO). Finally the samples were filtered using an Ultrafree-MC filter unit 100,000 (Millipore Co., Bedford, MA). The levels of 8-OHdG in each sample were quantified by high-performance liquid chromatography (HPLC) with electrochemical detection.

2.6. Semiquantitative RT-PCR assays for CYP450 isozymes and Ogg1 genes

Total RNA was isolated using an ISOGEN (Nippon Gene, Toyama, Japan) RNA extraction kit according to the manufacturer's protocol. cDNA was synthesized with an RNA LA PCR kit (Takara Bio Inc., Shiga, Japan) and amplified using a Rat cytochrome P450 competitive RT-PCR set (Takara Bio Inc., Shiga, Japan) according to the instructions provided. Cyclophilin was employed as an internal standard.

For detection of Ogg1 mRNA expression, cDNA was amplified with ampliTaq Gold polymerase (Applied Biosystems). The Ogg1 primers used were 5′ -ATC TGT TCC TCC AAC AAC AAC-3′ (forward) and 5′-GCC AGC ATA AGG TCC CCA CAG-3′ (reverse), amplifying a 504 bp product. The primer sequences of β-actin product used as an internal control were 5′ -ATC TGT TCC TCC AAC AAC AAC-3′ (forward) and 5′-GCC AGC ATA AGG TCC CCA CAG-3′ (reverse), amplifying a 281 bp product. The primers were amplified for 34 cycles with an annealing temperature 60 °C. PCR products were separated by 3.0% agarose gel electrophoresis, stained with ethidium bromide and analyzed using an FMBIO II Multi-View Image Analyzer Scanning Unit (Hitachi, Japan).

2.7. Statistical analysis

The statistical significance of differences between groups for each parameter was analyzed by a one-factor ANOVA test using Super ANOVA software (Abacus Concepts, Berkeley, CA, 1991). The Bonferroni–Dunnet test (control) and Fisher's protected least significant difference were applied as post hoc tests. Possible trends in the probabilities of relationship of number and area of GST-P positive foci, an end-point marker, total CYP450 content and NADPH-P450 reductase activity were applied by mixed model between Fisher's protected least significant difference test and Bartholomew's test [28].

3. Results

3.1. General observations and histopathological findings

α-BHC intake was estimated by average daily food consumption. The highest dose of α-BHC, 500 ppm, influenced on body and liver weights, on the contrary, the low dose of α-BHC, 1 ppm, significantly reduced liver weight (Table 1). Histological examination revealed liver foci of cellular alteration, mainly eosinophillic foci, was found in all rats. Centrilobular hypertrophy and tumors in the liver were observed only in the highest dose group. The mean number of all tumors per rat (adenomas and hepatocellular carcinomas) was 2.8±2.5 in the highest dose group, while no tumor was found in control and other α-BHC-treated groups.

Table 1
Final body and liver weights and α-BHC intake

Treatment	Dose (ppm)	No. of rats	Body weight		Liver weight		Estimated α-BHC intake (μg/kg bw/day)
			Initial (g)	Final (g)	Absolute (g)	Relative (%)	
DEN	0	12	112±6[a]	308±14	11.6±1.0	3.8±0.3	0
DEN→α-BHC	0.01	12	111±5	314±9	11.5±0.6	3.7±0.2	0.54
DEN→α-BHC	0.05	12	110±6	310±14	11.6±0.6	3.8±0.2	2.68
DEN→α-BHC	0.1	12	111±7	316±17	11.8±0.9	3.7±0.2	5.50
DEN→α-BHC	1	12	111±4	308±11	10.7±0.7[b]	3.5±0.2[b]	55.07
DEN→α-BHC	50	12	111±8	307±21	12.2±1.0	4.0±0.2	2845
DEN→α-BHC	500	12	109±6	282±10[b]	19.7±1.2[c]	7.0±0.5[c]	29899

[a] Mean±SD.
[b] Significantly different from the control group at $P<0.05$.
[c] Significantly different from the control group at $P<0.001$.

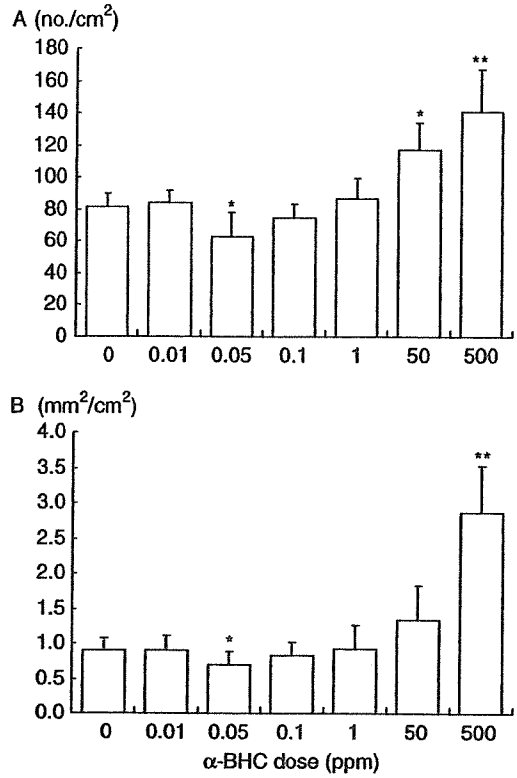

Fig. 1. Effect of α-BHC on induction of GST-P positive foci in rat livers. Rats were initiated with 100 mg/kg bw of DEN 3 times once a week and then promoted with various concentrations of α-BHC for 10 weeks. (A) Number and (B) area of GST-P positive foci. $*P<0.05$, $**P<0.01$ when compared with DEN alone group.

3.2. Immunohistochemistry of GST-P positive foci and PCNA

The number and/or area of GST-P positive foci were significantly increased in rats treated with 50 and 500 ppm of α-BHC when compared to the control value (Fig. 1). In contrast, at a low dose of 0.05 ppm, both number and area of GST-P positive foci were significantly inhibited when observed by Fisher's protected least significant difference test. Because of limitations of sample size and high variation of data, it was not surprising that the reduction of number and area of GST-P positive foci at 0.05 ppm of α-BHC showed a significant difference when applied in low power statistical method. However, the test for an increase in proportions, Bartholomew's test, of the number and area of GST-P positive foci in low dose, 0.05 ppm and in high doses, 50 and 500 ppm of α-BHC was significant ($P<0.05$) in determining a trend of dose-response curve. Thus, it might be suggested that the plotting concentration of α-BHC versus number and area of GST-P positive foci resulted in J-shaped dose-response relationships (Fig. 4A).

To measure cell proliferation in preneoplastic lesions, double staining of GST-P and PCNA was performed (Fig. 2A). The result revealed a significant increase in number of PCNA-positive cells within the area of GST-P positive foci in the

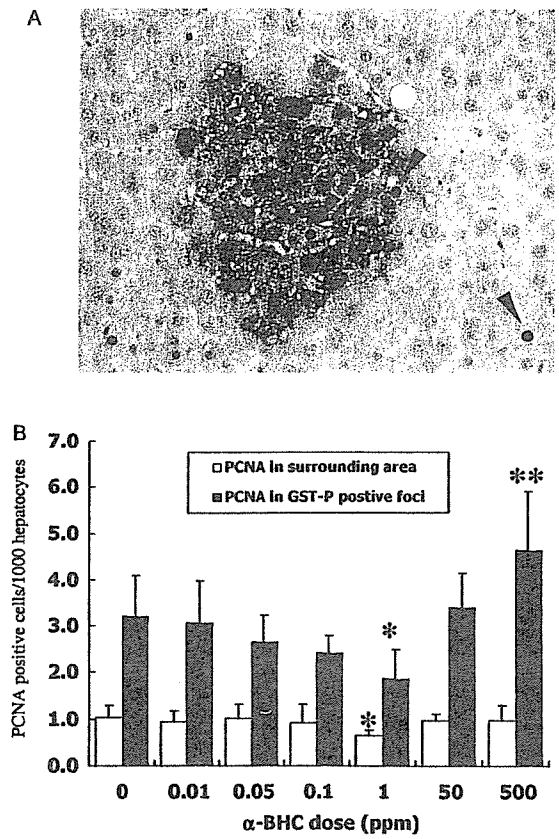

Fig. 2. Immunohistochemistry of double staining of GST-P and PCNA. (A) Photograph represents double staining of GST-P and PCNA, which PCNA was stained in brown color (triangle) and GST-P stained in red. Magnification 400× (B) Effect of α-BHC on number of PCNA in GST-P positive foci and surrounding area. $*P<0.05$, $**P<0.01$ when compared with DEN alone group.

500 ppm α-BHC-treated group (Fig. 2B). On the other hand, PCNA index within GST-P positive foci areas in the 1 ppm α-BHC dose group was significantly reduced. Similar tendencies were observed with the 0.05 and 0.1 ppm doses. The PCNA-positive cells in surrounding area, furthermore, were significantly decreased in rats fed 1 ppm of α-BHC.

3.3. Oxidative DNA damage and its repair and Phase I and II metabolism

As shown in Table 2, the 8-OHdG levels in liver were significantly lower than the DEN alone group with 0.1 or 1 ppm of α-BHC but increased with the 500 ppm dose. The expression level of Ogg1 mRNA showed a tendency to be augmented by treatment with 0.1 ppm α-BHC. The content of CYP450 in the liver was significantly reduced with 0.05 ppm, but dramatically raised at the highest dose. A similar pattern was observed for NADPH-P450 reductase activity. Using Batholomew's test, it was proved that increment of CYP450 content and NADPH-P450 reductase activity from low doses to high doses (0.05–500 ppm) are significantly different ($P<0.05$). Furthermore, GST activity considerably elevated in the highest dose group, but did not changed at low doses when compared with the DEN alone group.

Testosterone 2α-, 7α- and 16β-hydroxylase activities are diagnostic for CYP2C11, CYP2A1 and CYP2B1, respectively, whereas testosterone 2β- and 6β- hydroxylases are indicators of catalytic activity of CYP3A family members [24]. In Table 3, 16α-Testosterone metabolite is catalysed by CYP2B and 2C11. It was found that 50 and 500 ppm of α-BHC significantly enhanced the hydroxylation of 2β-, 6β- and 16β-testosterone. The highest concentration of α-BHC induced 7α-testosterone hydroxylase, whereas the hydroxylation of testosterone at the 2α-position was dramatically lowered. Interestingly, 2α- and 16α-hydroxylation was significantly elevated by 0.1 ppm of α-BHC illustrating an inverted J-shape for the dose dependence. In rats treated with 50 and 500 ppm of α-BHC, CYP2B1, 2C11/6, 2E1, 3A1/2 and NADPH-P450 reductase protein expression was increased more than 20-fold, 1.5–2-fold, 1.5-fold, 2–5-fold and 2–2.5 fold, respectively (Table 4). α-BHC at doses of 0.01–1 ppm did not cause alteration in CYP450 protein levels. The amount of CYP2B1 protein was undetectable level at low doses of α-BHC. In Fig. 3, mRNA expression of CYP2B1/2 was increased 2.5 and 8-fold in the 50 and 500 ppm α-BHC treated groups, respectively. On the other

Table 2
Effect of α-BHC on phase I and II xenobiotic metabolism, oxidative DNA damage and DNA reparing system in rat liver initiated with DEN

Treatment	8OHdG level (per 10^5 dG)	OGG1 mRNA expression (Ogg1/β-actin ratio)	Total P450 content (nmol/mg protein)	NADPH-P450 reductase activity (Umol/min/mg protein)	GST activity (U/mg protein)
DEN	0.258±0.039[a]	0.740±0.125	0.385±0.047	0.108±0.026	1.482±0.241
DEN→α-BHC 0.01ppm	0.221±0.016	0.815±0.191	0.363±0.039	0.102±0.022	1.421±0.126
DEN→α-BHC 0.05 ppm	0.242±0.030	0.851±0.230	0.309±0.035[b]	0.085±0.003[b]	1.463±0.178
DEN→α-BHC 0.1 ppm	0.197±0.033[b]	1.017±0.330	0.346±0.051	0.083±0.010	1.582±0.168
DEN→α-BHC 1 ppm	0.210±0.020[b]	0.857±0.096	0.384±0.056	0.091±0.012	1.409±0.135
DEN→α-BHC 50 ppm	0.281±0.036	0.841±0.262	0.480±0.049	0.114±0.011	1.643±0.342
DEN→α-BHC 500 ppm	0.528±0.056[c]	0.783±0.157	0.692±0.076[c]	0.194±0.028[c]	3.125±0.351[c]

[a] Mean±SD.
[b] Significantly different from the control group at $P<0.05$.
[c] Significantly different from the control group at $P<0.01$.

Table 3
Effect of α-BHC on hepatic microsomal CYP450 isozymes catalytic activities

Treatment	Testosterone hydroxylase activity (pmol/min/nmolP450)[a]					
	2α-	7α-	16α-	2β-	6β-	16β-
DEN	701.2±109.3[b]	57.9±17.3	1175.7±110.2	50.6±14.1	687.4±129.2	49.1±14.8
DEN→α-BHC 0.01ppm	717.4±64.9	54.0±14.4	1152.3±94.9	50.0±7.6	688.9±93.9	51.5±8.1
DEN→α-BHC 0.05 ppm	821.9±130.0	65.4±12.2	1325.4±220.4	52.6±10.2	651.4±26.1	45.4±8.7
DEN→α-BHC 0.1 ppm	933.9±75.6[c]	73.2±6.3	1501.7±124.9[c]	56.4±17.6	777.6±57.3	54.0±15.6
DEN→α-BHC 1 ppm	768.6±104.5	62.4±6.0	1275.4±162.3	68.8±5.8	832.3±70.7	65.8±7.1
DEN→α-BHC 50 ppm	654.7±50.0	67.0±6.5	1364.8±131.2	88.4±11.2[c]	893.3±92.7[c]	285.8±51.2[c]
DEN→α-BHC 500 ppm	293.0±47.4[d]	220.3±23.5[c]	1123.1±90.9	166.6±18.5[d]	908.6±89.7[c]	626.9±45.7[d]

[a] Regio- and stereo-selective hydroxylated testosterone metabolites were detected by HPLC.
[b] Mean±SD.
[c] Significantly different from the control group at $P<0.05$.
[d] Significantly different from the control group at $P<0.01$.

hand, CYP3A2 mRNA was induced 2-fold in 500 ppm group and tend to be decreased at 0.01 and 0.05 ppm, correlating with the protein level data. CYP2C11 mRNA expression did not change in the high dose treated groups but was significantly increased by 0.05 ppm α-BHC treatment.

4. Discussion

In the present experiment using GST-P positive foci as end-point markers in hepatocarcinogenesis, an inhibitory effect was found with α-BHC at low dose in the preneoplastic formation step of

Table 4
Effect of α-BHC on microsomal CYP450 isozymes protein expression in rat liver initiated with DEN

Treatment	CYP450 isozymes content (pmol CYP450/ mg protein)[a]				NADPH-P450 reductase[a] (μmol cytochrome C/ mg protein)
	2B1	2C11/6	2E1	3A2	
DEN	0	88.13±33.20	113.13±18.19	56.35±10.59	4.63±1.11
DEN→α-BHC 0.01 ppm	ND[b]	90.09±18.76	106.56±13.10	38.47±10.05	4.27±0.82
DEN→α-BHC 0.05 ppm	ND	89.11±10.29	134.18±38.80	39.21±7.04	4.34±0.76
DEN→α-BHC 0.1 ppm	ND	103.27±7.21	128.35±17.27	51.55±6.49	4.88±0.93
DEN→α-BHC 1 ppm	ND	108.49±27.70	147.60±31.45	55.85±9+96	4.86±0.96
DEN→α-BHC 50 ppm	60.49±18.59[c]	134.23±16.65[d]	169.35±33.86[d]	106.64±9.24[d]	7.86±1.62[c]
DEN→α-BHC 500 ppm	175.83±19.72[c]	189.02±58.97[c]	163.57±42.51[d]	285.10±66.42[c]	12.51±2.35[c]

[a] Mean±SD.
[b] ND, not detectable.
[c] Significantly different from the control group at $P<0.01$.
[d] Significantly different from the control group at $P<0.05$.

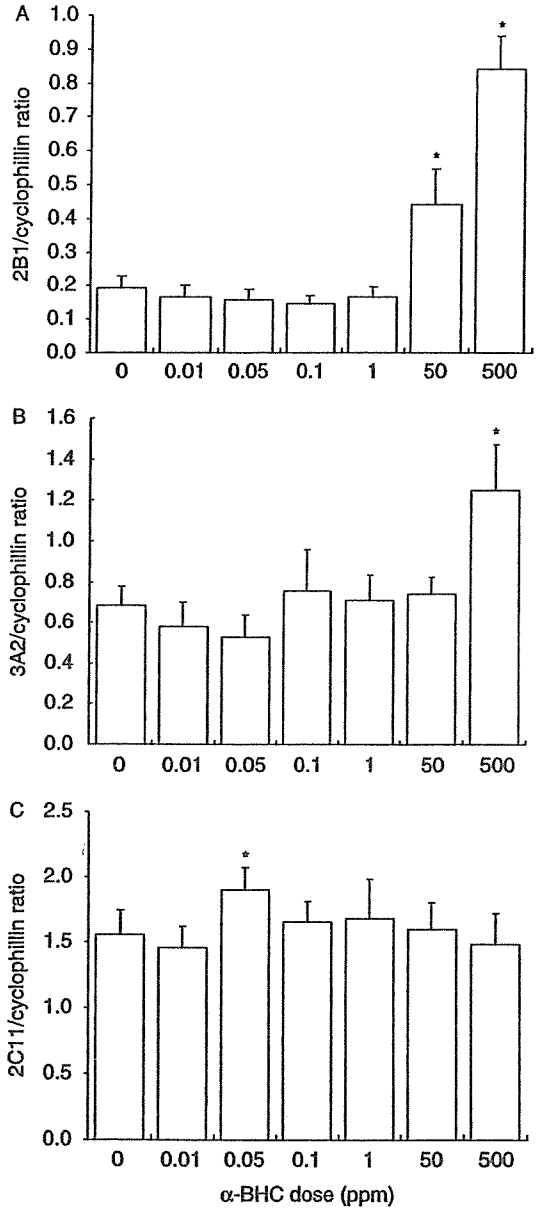

Fig. 3. Effect α-BHC on CYP450 isozymes mRNA expression in liver of rat initiated with 100 mg/kg bw of DEN 3 times once a week. (A) CYP2B1/2, (B) CYP3A2, (C) CYP2C11 detected by competitive RT-PCR. $^{*}P<0.05$, $^{**}P<0.01$ when compared with DEN alone group.

hepatocarcinogenesis of male F344 rats initiated with DEN. The dose response relationship for GST-P positive foci exhibited a J-shape curve, in line with the previous investigation of this chemical using the Ito test [5,6]. It has been accepted that there is the correlation between GST-P positive foci and liver tumor formation in the rat [29–32]. Thus in the rat, α-BHC possibly shows hormesis effect on early stages of DEN induced hepatocarcinogenesis.

Xenobiotics metabolizing enzymes (XMEs) play central roles in the metabolism and/or detoxification of xenobiotics. When α-BHC is introduced to the body, XMEs response in order to protect or defend the body against potentially harmful insults. For the body to minimize the insults caused by α-BHC, the liver is well equipped with a diverse assortment of XMEs including various Phase I and Phase II enzymes. A reduction in the number and area of GST-P positive foci in response to low doses of nongenotoxic carcinogens such as phenobarbital and dichlorodiphenyltrichloroethane (DDT) has been reported [15,17]. The molecular mechanism of this preneoplastic lesion development and its interrelation with the cytochrome P450 system, however, has not yet been identified. Diwan and his colleagues found that the induction of phase I and II enzymes tightly correlated with hepatocellular carcinoma development in rats [33]. We here demonstrated that the total CYP450 content and NADPH-P450 reductase activity displayed a biphasic dose–response similar to GST-P positive foci data. Activity of CYP450 proteins is dependent on the NADPH-P450 reductase that transfers electrons from NADPH to CYP450 enzymes during the P450 catalytic cycle [34]. Hence, one possible mechanism of hormesis phenomenon of α-BHC on the early stages of rat hepatocarcinogenesis, may include NADPH-P450 reductase and CYP450 enzymes system.

α-BHC is considered a phenobarbital-like inducer that enhances CYP2B1 and 3A2 expression at the protein level [5]. These isozymes induced free radicals in phenobarbital-treated rats [35]. Therefore, they might play a major role in α-BHC bioactivation and increase its toxicity due to its correlation with GST-P positive foci and DNA oxidative damage. The present study firstly found that high concentrations of α-BHC influence on CYP2B1 and 3A2 mRNA expression. The CYP induction by α-BHC showed a similar pattern to phenobarbital. The influence of α-BHC on the upstream pathway of these CYP isozymes is still unclear. It might be presumed that α-BHC at high dose influenced orphan nuclear receptors, constitutive

androstane receptors and pregnane X receptor, which regulate CYP2B1 and 3A2 transcription [36]. In case of low doses, however, a nonlinear threshold response with regard to CYP2B1 and 3A2 might result from multiple steps that necessary for 'turning on' of such genes. Transcription in general exhibits a burst-like pattern. Once chemical concentration is increased, the induction of transcription of such gene is rapidly generated [10].

CYP2C11, a major constitutive male-specific isoform, was here found to be up-regulated by low dose α-BHC treatment at the transcriptional level and with regard to catalytic activity detected by 2α- and 16α- testosterone metabolites. Thus, a plot of CYP2C11 activity against α-BHC concentration showed an inverted shape to the dose-response pattern of either GST-P or 8-OHdG (Fig. 4A,B). This suggests that CYP2C11 may play a role in the detoxification of α-BHC. The reduction in activity of CYP2C11at high dose of α-BHC may increase liver toxicity leading to the induction of oxidative stress and 8-OHdG formation.

GST plays as an important role in detoxifying α-BHC [37] and we here demonstrated a threshold in its activity to α-BHC at low doses. However, the GST family is composed of at least four classes, GST-A, GST-M, GST-P and GST-T and we could not determine which isoform predominated. Once we determined GST-P, a preneoplastic antigen over-expressed in chemical-induced tumors by a immuno-histochemical technique, hormesis phenomenon was overt.

The specific mechanism by which oxidative stress contributes to the development of carcinogenesis is largely unknown. One possible mechanism is the modulation of the expression of some genes involving growth signals and cell proliferation [38]. 8-OHdG, the most widely used marker for oxidative DNA damage, is considered to be involved in carcinogenesis in various experimental models [39]. The present study demonstrated increasing levels of 8-OHdG in high dose α-BHC-treated rats and reductions at low doses (Fig. 4B). Oxidative stress might influence the balance of cell homeostasis. A low dose of α-BHC tends to up-regulate Ogg1 mRNA expression. This might suggest not only Ogg1 but also the other DNA repair machinery play a role in removal of modified bases from DNA in this model [40].

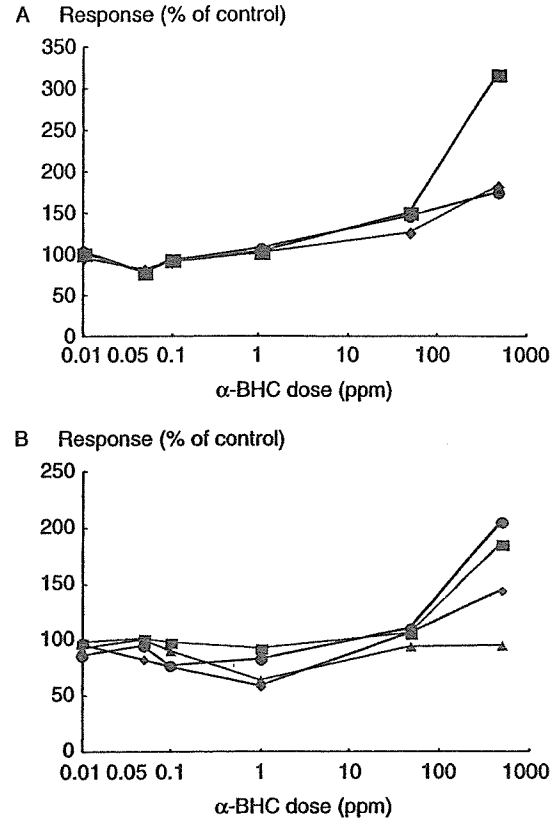

Fig. 4. Correlation of some biological markers in the liver of α-BHC treated rats. (A) P450 content, —◆—; number of GST-P-positive foci, —●—; area of GST-P-positive foci, —■—. B: 8-OHdG, —●—; PCNA positive foci in GST- positive foci, —◆—; PCNA positive foci in hepatocytes, —▲—; and relative liver weight, —■—. Hepatic effect is given as a percentage of control (DEN alone group).

Furthermore, the PCNA positive index in the GST-P positive foci decreased in the low dose group but increased in the high dose. This result is in agreement with TGF-β1 mRNA finding in which its expression was enhanced at low dose (data not shown). In general, toxicants tend to induce apoptosis at low exposure levels or early after exposure at high levels, where as they cause necrosis later at high exposure levels. TGF-β1 is one of the possible factors involving cell death and inducing apoptosis at least in early stages of carcinogenesis [41]. At low doses, α-BHC may trigger a cellular response leading to an increase

in apoptosis, then a decrease in cell proliferation follows and ultimately the clearance of cellular stresses. This homeostatic cellular response to low dose of α-BHC may overcompensate hepatocytes that inhibit the early stage of DEN induced hepatocarcinogenesis in rats.

Hormesis phenomenon seems to be concealed and cannot be explained by the change of one biological marker. To conserve homeostasis, when the target cells are exposed to chemicals, they might stimulate some remunerative machinery such as detoxifying and antioxidant systems, cell cycle regulation and programmed cell death. At very low doses of chemicals, the compensatory mechanism in target cells might recover cell injury so that not only a dose threshold but also a reduction in lesion development, as compared to the control case, may occur. Thus, hormesis may take place [42]. It was proposed that for DNA damaging carcinogens to produce a J-shape dose-response, the protective effect must overcompensate the genotoxicity as illustrated by the mutation rate [19]. With nongenotoxic carcinogens, there is no linear increase in DNA damage, so that a protective effect might more easily be observed. Thus, the J-shape dose-response curve of phenobarbital and 2,3,7,8-tetrachlorodibenzo-p-dioxin were observed not only in the case of rat liver foci formation but also liver tumor induction [15,43]. Each mechanism has its own dose-response pattern, which can be linear, threshold, J-shaped or otherwise. The dose-response of α-BHC for preneoplastic lesion formation is considered to be the result of an appropriate combination of the individual shapes of each biological marker. The method of statistical analysis applied also has influenced on the occurrence of hormesis [18]. By using the mixed model of two statistical analyses, we propose that the response curve for α-BHC at early stages of hepatocarcinogenicity probably features two parts, a J-shape at low doses which becomes linearly dose dependent at higher doses. It appears that using various biological markers for analyzing hormesis of α-BHC (Fig. 4), response points are best illustrate with varied concentrations plotted on a log scale [46]. The order of xenobiotic metabolism, Phase I and II enzymes, commonly occurs prior to oxidative stress and pathological changes, therefore, the concentration of α-BHC showing inhibitory effects on Phase I and II enzyme markers was lower, 0.05 ppm, than those of 8-OHdG, 0.1 and 1 ppm, cell proliferating index, 1 ppm, and liver weight, 1 ppm, in this model. This shift pattern was not only observed with α-BHC but also with β-BHC. β-BHC at low doses was resulted in a decrease of γ-glutamyltransferase foci and CYP450 activity in rat liver at different concentrations [44].

In summary, the present study showed hormesis effects of α-BHC on DEN induced hepatocarcinogenesis indicative of a dose threshold for its carcinogenicity. The possible mechanisms might involve xenobiotic metabolism and oxidative stress. Further study in a long-term experiment of α-BHC using tumor formation as the end-point is now under investigated.

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Review

Hormesis in Carcinogenicity of Non-genotoxic Carcinogens

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Abstract: Recently the idea of hormesis, a biphasic dose-response relationship in which a chemical exerts opposite effects dependent on the dose, has attracted interest in the field of carcinogenesis. With non-genotoxic agents there is considerable experimental evidence in support of hormesis and the present review highlights current knowledge of dose-response effects. In particular, several *in vivo* studies have provided support for the idea that non-genotoxic carcinogens may inhibit hepatocarcinogenesis at low doses. Here, we survey the examples and discuss possible mechanisms of hormesis with cytochrome P450 inducers, such as phenobarbital, 1,1-bis(*p*-chlorophenyl)-2,2,2-trichloroethane (DDT), α-benzene hexachloride (α-BHC), and other non-genotoxins. Epigenetic processes differentially can be affected by agents that impinge on oxidative stress, DNA repair, cell proliferation, apoptosis, intracellular communication and cell signaling. Non-genotoxic carcinogens may target nuclear receptors, cause aberrant DNA methylation at the genomic level and induce post-translational modifications at the protein level, thereby impacting on the stability or activity of key regulatory proteins, including oncoproteins and tumor suppressor proteins. Via multiple epigenetic lesions, non-genotoxic carcinogens can elicit a variety of changes contributing to cellular carcinogenesis. (J Toxicol Pathol 2006; **19**: 111–122)

Key words: hormesis, non-genotoxic carcinogen, threshold, oxidative stress

Exposure to Carcinogens and Human Cancer

The risk of cancer in humans is dependent on environmental, occupational and recreational exposure to carcinogens as well as on spontaneous events that reflect human variation in the efficiency or fidelity of various cancer-critical processes. Assessment of carcinogenic potential of agents to which human beings are exposed is clearly of prime importance but this is complicated by the existence of both genotoxic and non-genotoxic classes of chemical carcinogens, divided on the basis of their ability to react with DNA and form adducts. It is well established that genotoxic agents can covalently bind to DNA and increase the number of mutations, thereby causing errors in DNA replication. Positive data for chromosomal effects like aneugenicity or clastogenicity, in the absence of mutagenicity, may support separate characterization of compounds that exert carcinogenic effects only at high doses[1]. Non-DNA-reactive compounds, such as topoisomerase inhibitors[2,3] or inhibitors of the spindle apparatus or associated motor proteins[4–7] are considered to act by this mechanism[8].

Many chemicals that produce tumors in experimental animals have been shown to act by epigenetic mechanisms that do not necessarily involve DNA attack or hereditable genetic alteration[9]. The indirect nature of the mechanisms involved means that prolonged exposure to high levels of chemicals is necessary for the production of tumors[10]. With such non-genotoxic carcinogens, theoretically cancer would not occur at exposures below a threshold at which the relevant cellular effect is not operative. Also, in contrast to DNA-reactive genotoxic effects, epigenetic mechanisms may be unique to the rodent species used for testing. Certain chemical carcinogens have been well studied and provide examples for the use of mechanistic information in risk assessment. Non-genotoxic carcinogens including tumor promoters, like dioxin for example, do not bind directly to DNA but alter cell proliferation and physiology by inducing expression of enzymes involved in xenobiotic metabolism, DNA repair, methylation and cell signaling. An altered hormonal environment may enhance the rate of cell replication by mechanisms involving receptor-mediated processes without DNA-reactivity, thus increasing the likelihood of promotion/progression of spontaneously initiated cells[11].

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Threshold in Carcinogenicity of Environmental Carcinogens

With examination of the risk of human exposure to chemicals with carcinogenic potential in the environment, a natural question is whether a threshold exists for observed effects. Recently the concepts of "practical" and "perfect" thresholds for genotoxic and non-genotoxic compounds, respectively, have been proposed[8]. The idea is that carcinogens can be further classified as: (i) Genotoxic agents without a threshold in their effects; (ii) Genotoxic compounds for which the existence of a threshold is possible but is not yet sufficiently supported; (iii) Genotoxic carcinogens for which a "practical" threshold is supported by studies on mechanisms and/or toxicokinetics; (iv) Genotoxic carcinogens for which a "perfect" threshold is associated with a no-observed effect level (NOEL) and (v) Non-genotoxic carcinogens for which a "perfect" threshold is associated with a NOEL[8].

Until recently, risk assessment in the field of chemicals distinguished between two types of agents: the first comprising potentially toxic chemicals that may induce physical damage to human beings at above a certain threshold of exposure or intake[12]. The second class is believed to cause harm at any level above zero, even at very tiny doses (stochastic effects). However, the conventional view of toxicity and risk has been challenged by recent investigations pointing to potential beneficial effects of exposure to otherwise hazardous substances at very low dose levels. Most of the substances involved are non-genotoxic chemicals, acting as cytochrome P-450 inducers at high doses and exhibiting promoting effects on hepatocarcinogenesis in rodents, and the existence of a threshold was postulated for examples acting by epigenetic mechanisms, such as phenobarbital[13,14], α-benzene hexachloride (α-BHC)[15], 1,1-bis(*p*-chlorophenyl)-2,2,2-trichloroethane (DDT)[16], 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) or caffeic acid[17]. However, genotoxic carcinogens, such as 2-acetylaminofluorene (2-AAF)[18,19] and ionizing radiation[20] may also be included. Inhibitory effects of all these agents on carcinogenesis at low doses have been subsumed under the heading of hormesis[12].

The Hormesis Theory

Hormesis has been defined as a dose/response relationship in which there is a biological activation at low doses, but an inhibition at high doses, or vice versa, resulting in a U, J or inverted U-shaped dose response[21,22].

The history of hormesis originated in the laboratory of Prof. Hugo Schulz at the University of Greifswald in Northern Germany. He found that many agents appeared to stimulate metabolism at low concentrations but inhibit them at higher doses[21]. This provided a toxicological explanation for his development of homeopathic ideas. Interest in the effects of low doses rapidly expanded, especially with many studies of interactions involving (mainly) plants, bacteria and fungi, most notably in Europe, the USA and Japan[21]. Hormetic effects were observed at low exposure levels based on the dose-response pattern with data from developmental toxicity studies, indicating that there might actually be a reduced risk of toxic effects at low exposure levels[23]. Hormesis implies the existence of a threshold dose level and there are dose-response models that include parameters that account for the threshold.

With ionizing radiation, hormesis was interpreted to be due to adaptation to background radiation exposure, as well as metabolic protection against the array of other abiotic stresses in the environment[20,24]. Weak endogenous carcinogens, such as reactive oxygen species (ROS), as well as micronutrient deficiencies and environmental toxins are obvious causes of non-radiation induced DNA damage which might lead to oncogenic transformation in non-irradiated cells[25]. The results suggested that at the level of background radiation, various forms of non-radiation DNA damage in tissues occur to much higher extents than those due to the low-dose radiation exposure. It has been proposed from the published data that mammalian cells have the physiological capacity to protect themselves constantly by preventing and repairing DNA damage. Furthermore, damaged cells are susceptible to removal by apoptosis or the immune system and chronic low-dose-rate radiation activate the immune system of the whole body[26]. Low-dose radiation was suggested to induce cellular signaling that may stimulate cellular protection systems over hours to weeks. Enhanced and persistent protective responses might reduce the steady state level of non-radiation DNA damage, thereby impacting on deleterious outcomes such as cancer and aging[25].

Hormetic Effects in Carcinogenesis

The question whether the concept of hormesis can be generalized to carcinogenesis has been recently discussed[27-29]. E. Calabrese and L. A. Baldwin cite numerous examples in well-designed studies providing evidence for U- and J-shape dose relationships with respect to different biomarkers of carcinogenesis in different animal models. For some chemicals tested, carcinogens were found to be similar to other toxicants in improving the outcome at low doses, although the mechanisms of their action remained unclear. Therefore, it appears very important to answer the question of how carcinogens act at low doses. Early stage carcinogenesis includes initiation with the occurrence of DNA damage and adaptive DNA repair. In 1983, Camurri *et al.*[30] observed a decrease of chromosomal aberrations with low dose styrene treatment. The response of human keratinocytes to a low dose of the well-known methylating agent, N-methyl-N'-nitro-N-nitrosoguanidine, was studied by Kleczkowska and Althaus[31]. It was found that at concentrations in the 0.05 to 50 nM range DNA unwinding and DNA strand breaks were significantly reduced, while at high doses they were enhanced compared to the control case. Inhibition activity regarding DNA damage at low doses was explained by activation of poly(ADP)-ribose. Furthermore,

assessment of the effects of Hg^{2+} on O^6-methylguanine-DNA methyltransferase activity of human buccal fibroblasts by Liu *et al.*[32] revealed elevation at low doses of 0.3 to 3 μM. With the dose-response curves of rat hepatic DNA damage for different types of carcinogens assessed by Kitchen and Brown[33], 11 showed non-monotonic character with some treated values lower than in controls.

The promotion stage of carcinogenesis has also been studied in the low dose range with regard to various parameters of interest. Examples include cell turnover with caffeic acid in the rat forestomach and kidney, altered hepatic foci formation with TCDD in diethylnitrosamine (DEN)-pretreated partially hepatechtomized rats[17], and urinary bladder hyperplasia in saccharin-treated rats[34]. Several chronic bioassays for carcinogenicity in rats and mice have demonstrated a negative correlation between proliferative hepatocellular lesions and lymphomas at low and medium dose levels[35]. In addition, TCDD at hepatocarcinogenic doses was reported to be capable of causing dose-dependent reduction in mammary and uterine tumors[36]. In 1994, Cook[37] reported that dioxin-treated rats displayed substantial decrease in tumors of the adrenals and pancreas and more modestly, in the liver. Examples of hormesis also include TCDD-mediated reduction in tumor incidence after exposure to low doses of radiation[20] or metals such as selenium[38]. U-shape responses were also observed for chemically induced pulmonary tumors[39–41] and testicular cancer[42].

Threshold in Phenobarbital Hepatocarcinogenicity

Recently, especial attention has been devoted to the carcinogenicity of low doses of phenobarbital, a sedative and anticolvulsant, which is used widely for long-term clinical therapy. It is also a well-known non-genotoxic carcinogen and tumor promoter in rodents. Epidemiological studies have not shown phenobarbital-related tumors in humans, indicating that humans may have low sensitivity to toxic effects of phenobarbital. In the rat, Goldsworthy *et al.*[43] reported no promotion by phenobarbital below 10 ppm with regard to the enzyme-altered foci. Furthermore, Kitagawa *et al.*[44] found inhibitory effects of both phenobarbital and another tumor promoter, DDT, on carcinogenesis when given together with relatively high doses of carcinogens. Similarly, Pitot *et al.*[45] found a slight decrease of altered hepatic foci by 10 ppm phenobarbital, and Maekawa *et al.*[46] demonstrated similar results with 1 ppm phenobarbital. To determine the practical threshold level for hepatopromoting effects of phenobarbital, Kitano *et al.*[13] investigated dose dependence using a rat liver medium-term bioassay (Ito test)[47]. When phenobarbital was administered to rats in a wide range of doses of 0.01 to 500 ppm in the diet for 6 weeks after a single intraperitoneal injection of DEN in serial experiments, glutathione S-transferase placental form (GST-P) positive foci, preneoplastic lesions in the liver, were found to be increased dose dependently in rats given 60–500 ppm. However, with doses in the range of 1–7.5 ppm, decrease was evident as compared to the control group, this being statistically significant at 1 and 2 ppm (Fig. 1). It was concluded that phenobarbital effects reflect hormesis in the rat liver, indicating the existence of a threshold for its carcinogenicity, suggested to be related to inhibition of cytochrome P-450 CYP3A2 protein expression by low doses of the chemical[13].

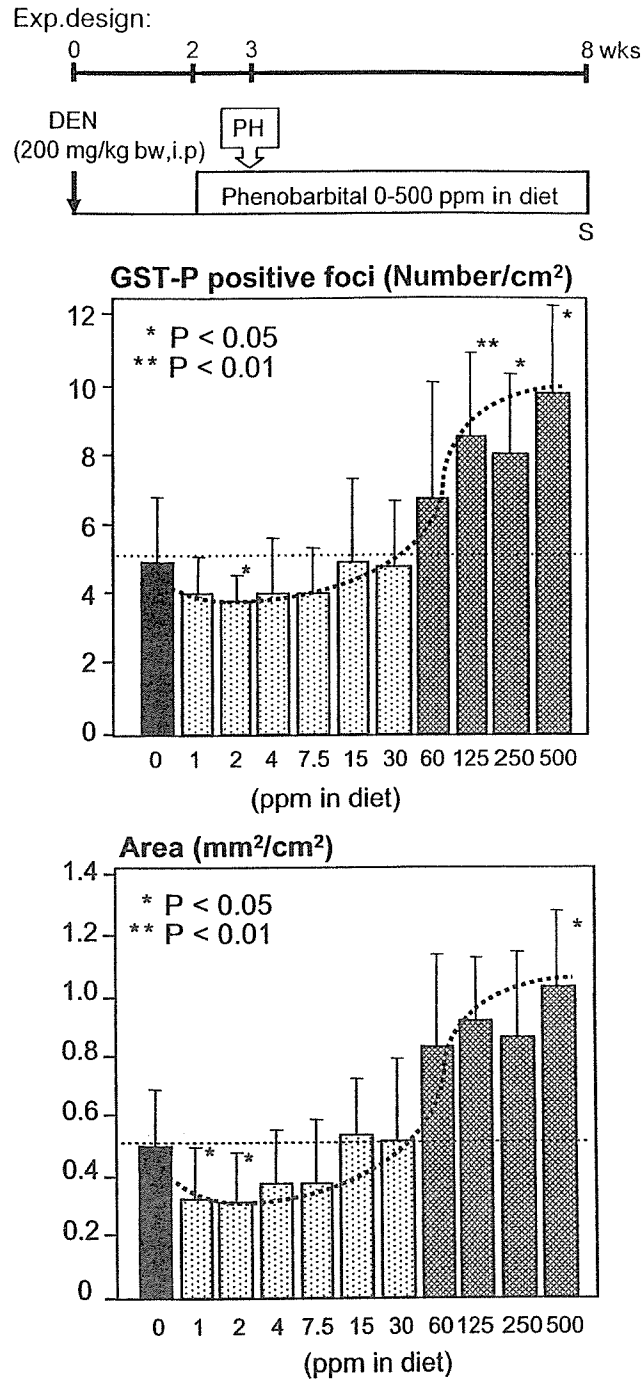

Fig. 1. Induction of GST-P positive foci in the livers of rats treated with phenobarbital in a medium-term bioassay (Ito test). PH, 2/3 partial hepatectomy. S, sacrifice.

For further clarification of the hormetic influence of

phenobarbital, Kinoshita *et al.*[14] investigated doses of 0, 2, 15 and 500 ppm applied in diet to male F344 rats for 10 or 33 weeks after initiation of hepatocarcinogenesis with DEN. Formation of GST-P positive foci and liver tumors was inhibited at 2 ppm after 10 and 33 weeks of phenobarbital administration, respectively (Fig. 2). Histopathological examination further demonstrated a significant reduction in the multiplicity of total tumors, in particular, hepatocellular carcinomas (HCCs), and a tendency for decreased incidences of HCCs and adenomas at 2 ppm[14]. In contrast, high dose administration resulted in strong elevation of HCC and total tumor multiplicities, this appearing to be related to increased generation of hydroxyl radicals, a marker of oxidative damage 8-OHdG, CYP2B1/2 and CYP3A2 mRNAs and the protein level, activity, and gene expression of other Phase I and II xenobiotic metabolizing enzymes. Inhibition at low doses was considered to be due to the suppression of 8-OHdG generation and cellular proliferation within areas of GST-P positive foci, as well as programmed cell death, apoptosis, in background liver parenchyma. The decrease of 8-OHdG levels induced by phenobarbital at low dose was possibly a result of elevated expression of the gene encoding the enzyme responsible for the repair of 8-OHdG lesions, oxoguanine glycosylase 1 (Ogg1). The reduction of apoptosis in the normal-appearing liver tissue surrounding the GST-P positive foci, which might have been due to the inhibition of oxidative DNA damage, was suggested to suppress enlargement of foci because of elevated sensitivity to stimuli for regeneration[14]. Another explanation for the suppressive effect of phenobarbital on the development of preneoplastic lesions might involve stimulation of hepatic drug-metabolizing enzymes, which detoxify carcinogens[45]. Activation of P-450 isoenzymes CYP2C11 and NADPH-cytochrome P-450 reductase (OR) in liver microsomes observed after administration of phenobarbital at low dose, if not accompanied by elevation of their protein expression leading to the generation of large amount of hydroxyl radical OH, might have a protective effect[14]. The available results thus indicate that the compound exhibits hormetic effects on rat hepatocarcinogenesis initiated with DEN by differentially altering cell proliferation, apoptosis and oxidative DNA damage at high and low doses.

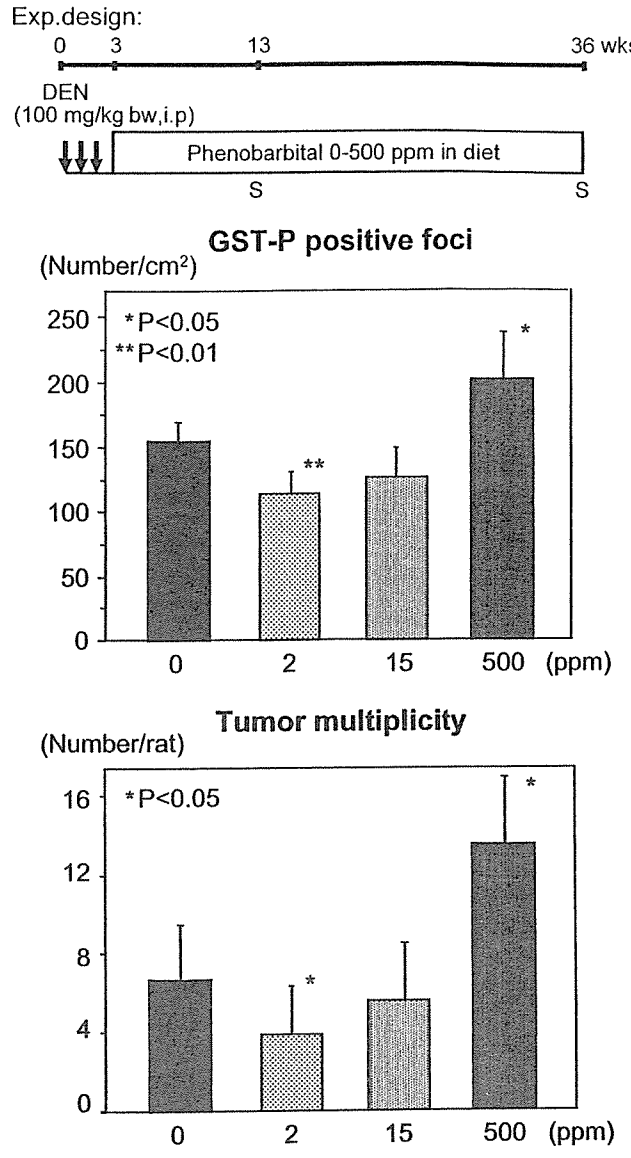

Fig. 2. Hepatocarcinogenicity of phenobarbital in the rat liver: GST-P positive foci and tumor development (DEN→PB). S, sacrifice.

Dose-response for α-benzene hexachloride hepatocarcinogenicity

α-BHC, a major organochlorine byproduct in the manufacture of lindane (γ-BHC), has been used in admixtures with lindane for agricultural purposes. The α-isomer of BHC has been categorized as a non-genotoxic carcinogen because of induction of liver tumors in rodents after high dose administration in the long-term but no mutagenicity in the Ames test. 2,4,6-trichlorophenol is the major metabolite in α-BHC metabolism by the cytochrome P-450 oxidoreductase system. After dechlorination and dehydrochlorination of α-BHC, removable chlorine atoms might react with hydrogen peroxide to produce hypochlorous radicals binding to DNA and formation of chlorinated DNA adducts, like 8-chloro-2-deoxyguanosine, 5-chloro-2-deoxycytidine and 8-chloro-2-deoxyadenosine[48,49]. Hyperplastic nodules and carcinomas in the livers of rats and mice were found induced by the long-term administration of high doses of α-BHC (such as 500 or 1000 ppm), but not β- and γ-BHC[50,51]. Furthermore, early toxicological studies revealed that α-, β-, and γ-BHC are potent inducers of hepatic monooxygenases in rats[52], in addition to causing liver enlargement[53,54]. Since induction of the monooxygenase system is assumed to influence the promotion stage[55,56], the mechanism of α-BHC carcinogenicity is likely to be due to its influence on spontaneously initiated hepatocytes[50,51].

To search whether α-BHC exhibits hormesis regarding