Original Article

Lack of Hepatocarcinogenicity of Combinations of Low Doses of 2-amino-3, 8-dimethylimidazo[4,5-*f*]quinoxaline and Diethylnitrosamine in Rats: Indication for the Existence of a Threshold for Genotoxic Carcinogens

Min Wei[1], Anna Kakehashi[1], Shotaro Yamano[1], Seiko Tamano[2], Tomoyuki Shirai[3], Hideki Wanibuchi[1]* and Shoji Fukushima[1,4]*

[1] Department of Pathology, Osaka City University Graduate School of Medicine, 1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585, Japan

[2] DIMS Institute of Medical Science, Inc., 64 Goura, Nishiazai, Azai-cho, Ichinomiya 491-0113, Japan

[3] Department of Experimental Pathology and Tumor Biology, Nagoya City, University Graduate School of Medical Sciences, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan

[4] Japan Bioassay Research Center, 2445 Hirosawa, Hadano, Kanagawa 257-0015, Japan

Abstract: The purposes of the present study were to evaluate the hepatocarcinogenicity of concurrent treatment of 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) and diethylnitrosamine (DEN) in rats and to determine whether no effect levels of combinations of these two different structural categories of genotoxic hepatocarcinogens exist. Two 16-week rat hepatocarcinogenesis assays were performed using a total of 790 male F344 rats. In experiment 1, we evaluated the effects of concurrent treatment of a subcarcinogenic dose of DEN on rat hepatocarcinogenesis induced by various doses of MeIQx. In experiment 2, we determined hepatocarcinogenicities of combinations of MeIQx and DEN at subcarcinogenic doses, low carcinogenic doses and high carcinogenic doses. Quantitative analyses of glutathione *S*-transferase placental form (GST-P)-positive foci, a preneoplastic lesion of the liver in rats, revealed that concurrent treatment with subcarcinogenic doses of DEN did not enhance MeIQx-induced rat hepatocarcinogenicity. We also found that concurrent treatment with combinations of subcarcinogenic doses of DEN and MeIQx was not hepatocarcinogenic, indicating that the combined effects of subcarcinogenic doses of DEN and MeIQx were neither additive nor synergistic. Moreover, concurrent treatment with low carcinogenic doses of these 2 carcinogens did not show additive or synergistic effects. Synergetic effects were observed only in rats coadministered high carcinogenic doses of the 2 carcinogens. These results demonstrate the existence of no effect levels of combinations of these 2 genotoxic hepatocarcinogens, and provide new evidence supporting our idea that there is a threshold, at least a practical threshold, that should be considered when evaluating the risk of genotoxic carcinogens. (DOI: 10.1293/tox.25.209; J Toxicol Pathol 2012; 25: 209–214)

Key words: MeIQx, DEN, concurrent treatment, carcinogenic threshold, low dose carcinogenicity

Introduction

Exposure to environmental carcinogens is one of the most significant causes of human cancers. Determination of the dose-response relationship between carcinogen exposure and induction of cancer is one of the most important areas of chemical risk assessment. It is generally assumed that genotoxic carcinogens exert a non-threshold carcinogenic effect.

This concept, however, is being challenged, as advancements in the understanding of the molecular mechanisms of carcinogenesis are being made and experimental evidence continues to accumulate showing that individual genotoxic carcinogens do not exert mutagenic or carcinogenic effects at low doses[1–6]. Given the probability that humans are exposed concurrently or sequentially to trace concentrations of multiple environmental carcinogens, examination of the effects of combinations of low doses of genotoxic carcinogens is an indispensable part of cancer risk assessment.

Previous studies of carcinogenic responses in rats exposed simultaneously to multiple carcinogens indicated that additive or synergistic effects were usually evident at high exposure levels of carcinogens, but this was not always the case at low carcinogen levels (reviewed by Shirai T[7]). For example, concurrent treatment with low doses of 3 *N*-nitro-

so compounds (genotoxic carcinogens) did not show additive effects on liver tumor development; only treatment with high doses exerted an additive effect[8]. Furthermore, concurrent treatment with low doses of 5 or 10 heterocyclic amines did not enhance development of preneoplastic lesions of the liver either additively or synergistically when given at the post-initiation stage in medium-term live bioassays (Ito test)[9–11]. These findings suggested that concurrent treatment with genotoxic carcinogens at low doses did not necessarily entail additive risk for carcinogenicity[7].

The effects of combinations of different structural categories of genotoxic carcinogens, e.g., combinations of heterocyclic amines and *N*-nitroso compounds, have not been evaluated. It also should be noted that the studies mentioned above focused mainly on the enhanced carcinogenic effects of combinations of multiple carcinogens and did not address the issue of threshold. Using various carcinogenesis models in different rat strains, we have demonstrated the existence of no effect levels for the hepatocarcinogenicity of 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), a genotoxic heterocyclic amine contained in seared fish and meat, and also for diethylnitrosamine (DEN), a genotoxic *N*-nitroso compound synthesized in the stomach through the reaction of secondary amines and nitrites in the diet[1]. The purposes of the present study were to evaluate the hepatocarcinogenicity of the concurrent treatment of MeIQx and DEN in rats and to determine whether no effect levels of combinations of these two genotoxic hepatocarcinogens exist.

Materials and Methods

Chemicals and diets

MeIQx (purity, 99.9%) was purchased from the Nard Institute (Nishinomiya, Japan), and DEN (purity>99.5%) was purchased from Sakai Research Laboratory (Fukui, Japan). Basal diet (powdered MF, Oriental Yeast Co., Ltd., Tokyo, Japan) and diets containing MeIQx were prepared once a month by Oriental Yeast Co., Ltd., Japan.

Animals

A total of 790 male F344 rats were supplied from Charles River Laboratories Japan, Inc. (Shiga, Japan) and were used at 21 days of age. Animals were housed in polycarbonate cages (5 rats/cage) in experimental animal rooms with a targeted temperature of 22 ± 3 °C, relative humidity of 55 ± 5% and a 12-h light/dark cycle. Diet and tap water were available *ad libitum* throughout the study. Fresh diet and drinking water were supplied to the animals twice weekly. Body weights, food consumption and water intake were measured weekly.

Experimental protocols

Animal experiment protocols were approved by the Institutional Animal Care and Use Committee of Osaka City University Medical School.

Experiment 1 was designed to evaluate the effects of subcarcinogenic doses of DEN on MeIQx-induced rat hepatocarcinogenesis. A total of 400 rats were randomized into 12 groups and treated with test chemicals for 16 weeks as shown in the Table 1. Group 1 was the control group without administration of any of test compounds. Groups 2–6 were MeIQx-alone treatment groups that were fed diets containing MeIQx at doses of 0.01, 0.1, 1, 10, and 100 ppm, respectively. Group 7 was the DEN-alone treatment group that was administered 0.01 ppm DEN in the drinking water. Groups 8–12 were the concurrent treatment groups coadministered the above doses of MeIQx and 0.01 ppm DEN. MeIQx dose selection was based on the results of a previous low-dose MeIQx hepatocarcinogenicity study; in that study, hepatocarcinogenic effects were not observed at doses of 1 ppm MeIQx and below[12]. The dose of 0.01 ppm for DEN was the maximum noncarcinogenic dose observed in a previous low-dose DEN hepatocarcinogenicity study[12]. The treatment period of 16 weeks was the same as in our previous low-dose carcinogenicity studies[1]. All rats were killed at the end of week 16 under deep anesthesia. At necropsy, livers were excised and weighed, and then 3 slices each from the left lateral, medial, and right lateral lobes were cut and placed in 10% phosphate buffered formalin. Following fixa-

Table 1. Final Body and Liver Weights, and Intakes of Test Chemicals (Experiment 1)

Group	MeIQx (ppm)	DEN (ppm)	No. of rats	Total intake of MeIQx (mg/kg bw)	Total intake of DEN (mg/kg bw)	Body weight (g)	Liver weights Absolute (g)	Liver weights Relative (%)
1	—	—	40	0	0	321 ± 13	9.54 ± 0.54	2.97 ± 0.11
2	0.01	—	40	0.07	0	332 ± 15 [a]	9.79 ± 0.59	2.95 ± 0.11
3	0.1	—	40	0.71	0	338 ± 16 [a]	9.83 ± 0.63	2.91 ± 0.09
4	1	—	40	7.11	0	332 ± 14 [a]	9.76 ± 0.66	2.94 ± 0.13
5	10	—	30	71.82	0	332 ± 17 [a]	9.94 ± 0.64 [a]	2.99 ± 0.10
6	100	—	10	720.79	0	325 ± 21	11.17 ± 0.88 [a]	3.44 ± 0.08 [a]
7	—	0.01	40	0	0.095	327 ± 15	9.67 ± 0.58	2.95 ± 0.09
8	0.01	0.01	40	0.07	0.093	344 ± 18 [a,b]	9.80 ± 0.59 [a]	2.85 ± 0.08 [a,b]
9	0.1	0.01	40	0.69	0.093	340 ± 16 [a,b]	9.79 ± 0.61	2.88 ± 0.10 [a,b]
10	1	0.01	40	6.86	0.095	341 ± 21 [a,b]	9.84 ± 0.74 [a]	2.89 ± 0.12 [a,b]
11	10	0.01	30	70.42	0.095	326 ± 14	9.78 ± 0.70	2.99 ± 0.13
12	100	0.01	10	704.20	0.093	323 ± 14	11.01 ± 0.48 [a,b]	3.41 ± 0.05 [a,b]

—, 0 ppm in the diet or drinking water. [a] Significantly different from group 1. [b] Significantly different from the respective MeIQx-alone group.

tion, liver tissues were embedded in paraffin and processed for histopathological examination and immunohistochemical analysis of glutathione *S*-transferase placental form (GST-P)-positive foci, a well-established preneoplastic liver lesion in rats that can extend the range of the observable effect levels of a carcinogen and has been accepted as a reliable and sensitive end-point marker in assessment of the carcinogenic effects of environmentally relevant concentrations of carcinogens[1,7,13].

Experiment 2 was designed to evaluate the hepatocarcinogenicities of combinations of MeIQx and DEN at subcarcinogenic doses, low carcinogenic doses and high carcinogenic doses. A total of 390 male F344 rats were randomized into 16 groups and treated with test chemicals for 16 weeks as shown in Table 3. Group 1 was the control group without administration of any test compounds. Groups 2–6 were MeIQx-alone treatment groups that were administered increasing doses of MeIQx as in Experiment 1. Groups 7–11 were DEN-alone treatment groups that were administered DEN in the drinking water at doses of 0.0001, 0.001, 0.01, 0.1 and 1 ppm. The DEN dosages in this study were the same as used in previous low-dose DEN hepatocarcinogenicity studies[12]. Groups 12–16 were coadministered MeIQx and DEN as follows: subcarcinogenic dose combination groups were administered 0.01 ppm MeIQx + 0.0001 ppm DEN (group 12), 0.1 ppm MeIQx + 0.001 ppm DEN (group 13) or 1 ppm MeIQx + 0.01 ppm DEN (group 14); the low carcinogenic dose combination group was administered 10 ppm MeIQx + 1 ppm DEN (group 15); and the high carcinogenic dose combination group was administered 100 ppm MeIQx + 10 ppm DEN (group 16). At the end of week 16, all rats were necropsied, and livers were processed and analyzed as in Experiment 1.

Examination of GST-P-positive foci in the liver

Anti-rat GST-P polyclonal antibody (Medical and Biological Laboratories Co., Ltd., Nagoya, Japan) at a dilution of 1:1000 was used for immunohistochemical staining of GST-P. GST-P-positive hepatocellular foci composed of 2 or more cells were counted under a light microscope[2,12,14,15]. Total areas of livers were measured using an IPAP color image processor (Sumica Technos, Osaka, Japan), and the number of GST-P-positive foci per square centimeter of liver tissue was calculated.

Statistical analysis

All mean values are reported as means ± SD. Statistical analyses were performed using the StatLight program (Yukms Co., Ltd., Tokyo, Japan) as described previously[2,14,16]. Briefly, homogeneity of variance was tested by the F test or Bartlett's test. Differences in mean values between the control and MeIQx- or DEN-alone treatment groups were evaluated by the 2-tailed Dunnett test when the variance was homogeneous and the 2-tailed Steel test when the variance was heterogeneous. Differences in mean values between the MeIQx- or DEN-alone groups and their respective concurrent treatment groups, and between concurrent treatment

groups and the control group were evaluated by the 2-tailed Student's test when the variance was homogeneous and the 2-tailed Welch's *t* test when the variance was heterogeneous. P values less than 0.05 were considered significant.

Results

Experiment 1

The final average body and liver weights and test chemical intakes are summarized in Table 1. In the MeIQx-alone treatment groups, the final body weights of the 0.01, 0.1, 1 and 10 ppm MeIQx groups were slightly but statistically significantly higher than that of the control group (non-treatment group). Consequently, a significant increase in absolute liver weights but not the relative liver weights was observed in the 10 ppm MeIQx group compared with the control group. There was an apparent treatment-related increase in liver weight in the 100 ppm MeIQx group, as evidenced by the findings that the final body weights of this group did not significantly differ from that of the control group but both the absolute and relative liver weights were significantly increased. There were no significant differences in body weight or absolute or relative liver weight between the MeIQx-alone groups and their respective MeIQx + 0.01 ppm DEN groups. Since 0.01 ppm DEN (group 7) had no effect on body or liver weight compared with the control group, the changes in body and liver weights observed in the MeIQx + 0.01 ppm DEN groups compared with the control groups were attributed to MeIQx treatment. The intake of DEN was similar among DEN treatment groups, and the intake of MeIQx was proportional to the administered doses (Table 1). No histopathological changes were observed in the livers of any of the groups.

Total numbers of GST-P-positive foci, composed of 2 or more cells (Fig. 1), per unit area of the rat liver are shown in Table 2. In the MeIQx-alone treatment groups, the numbers of GST-P-positive foci in the livers of groups administered 0.01 ppm to 10 ppm of MeIQx did not differ from the non-treatment control value. In contrast, a significant increase was observed in the group administered 100 ppm MeIQx. As expected, 0.01 ppm DEN did not increase the number of GST-P-positive foci; in fact, the number of GST-P-positive foci in this group showed a slight but significant decrease compared with the control group. There were no significant differences in the numbers of GST-P-positive foci between the groups receiving combinations of MeIQx and 0.01 ppm DEN and their respective MeIQx-alone groups. Importantly, the numbers of GST-P-positive foci in the livers of the groups receiving combinations of 0.01 ppm to 10 ppm of MeIQx and 0.01 ppm DEN did not differ from the control value. These results indicate that the subcarcinogenic dose of 0.01 ppm DEN did not exert either additive or synergistic effects on MeIQx-induced hepatocarcinogenesis in rats.

Experiment 2

The final average body and liver weights and test chemical intakes are summarized in Table 3. MeIQx-alone
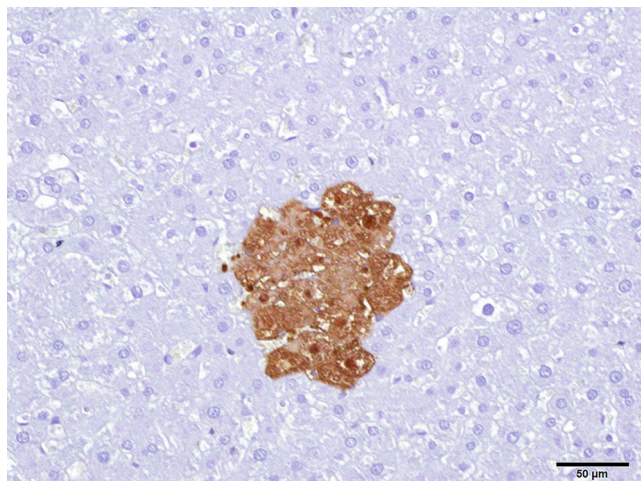
Fig. 1. GST-P-positive focus in the liver of a rat administered 100 ppm MeIQx + 1 ppm DEN (Experiment 2).

Table 2. GST-P-positive Foci in the Rat Liver (Experiment 1)

Group	MeIQx (ppm)	DEN (ppm)	No. of rats	GST-P-positive foci (no./cm^2)
1	—	—	40	0.11 ± 0.23
2	0.01	—	40	0.08 ± 0.18
3	0.1	—	40	0.10 ± 0.20
4	1	—	40	0.15 ± 0.24
5	10	—	30	0.17 ± 0.23
6	100	—	10	10.7 ± 3.97 [a]
7	—	0.01	40	0.03 ± 0.12 [a]
8	0.01	0.01	40	0.05 ± 0.14
9	0.1	0.01	40	0.10 ± 0.20
10	1	0.01	40	0.14 ± 0.26
11	10	0.01	30	0.16 ± 0.27
12	100	0.01	10	12.21 ± 7.07 [a, b]

—, 0 ppm in the diet or drinking water. [a] Significantly different from group 1. [b] Significantly different from the 100 ppm MeIQx-alone group.

Table 3. Final Body and Liver Weights, and Intakes of Test Chemicals (Experiment 2)

Group	MeIQx (ppm)	DEN (ppm)	No. of rats	Total intake of MeIQx (mg/kg bw)	Total intake of DEN (mg/kg bw)	Body weight (g)	Liver weights Absolute (g)	Liver weights Relative (%)
1	—	—	30	0	0	326 ± 13	8.99 ± 0.43	2.76 ± 0.10
2	0.01	—	30	0.06	0	327 ± 33	8.90 ± 0.99	2.72 ± 0.11
3	0.1	—	30	0.58	0	335 ± 15	9.30 ± 0.46 [a]	2.77 ± 0.09
4	1	—	30	5.65	0	330 ± 19	9.08 ± 0.62	2.75 ± 0.12
5	10	—	20	61.58	0	332 ± 12	9.47 ± 0.43 [a]	2.85 ± 0.11 [a]
6	100	—	10	623.29	0	326 ± 9	10.40 ± 0.48 [a]	3.19 ± 0.09 [a]
7	—	0.0001	30	0	0.001	319 ± 14	8.87 ± 0.49	2.78 ± 0.16
8	—	0.001	30	0	0.010	324 ± 15	9.20 ± 0.55	2.84 ± 0.09 [a]
9	—	0.01	30	0	0.096	315 ± 27	8.90 ± 0.74	2.83 ± 0.14
10	—	0.1	20	0	0.937	322 ± 15	9.17 ± 0.63	2.85 ± 0.15
11	—	1	10	0	10.220	317 ± 12	9.16 ± 0.55	2.89 ± 0.09 [a]
12	0.01	0.0001	30	0.06	0.001	340 ± 13 [a, b]	9.13 ± 0.46 [b]	2.69 ± 0.09 [a, b]
13	0.1	0.001	30	0.58	0.010	336 ± 13 [a, b]	9.40 ± 0.58 [a]	2.80 ± 0.12
14	1	0.01	30	5.60	0.097	334 ± 14 [a, b]	9.18 ± 0.51	2.75 ± 0.08 [b]
15	10	0.1	20	59.61	0.999	333 ± 27	9.55 ± 0.86 [a]	2.87 ± 0.09 [a]
16	100	1	10	625.68	9.655	331 ± 12 [b]	10.55 ± 0.72 [a, b]	3.19 ± 0.12 [a, b]

—, 0 ppm in the diet or drinking water. [a] Significantly different from group 1. [b] Significantly different from the respective DEN-alone group.

and DEN-alone treatments had no effect on body weights compared with the control group. No body weight suppression was observed in any combination treatment group. The final body weights of the 0.01 ppm MeIQx + 0.0001 ppm DEN, 0.1 ppm MeIQx + 0.001 ppm DEN, 1 ppm MeIQx + 0.01 ppm DEN and 100 ppm MeIQx + 1 ppm DEN treatment groups were significantly higher than their respective DEN-alone groups. Apparent treatment-related increases in both absolute and relative liver weights were observed in the groups administered 10 and 100 ppm MeIQx-alone and the groups administered 10 ppm MeIQx + 0.1 ppm DEN and 100 ppm MeIQx + 1 ppm DEN. The intake of DEN and MeIQx was proportional to the administered doses (Table 3). No histopathological changes were observed in the livers of any of the groups.

The numbers of GST-P-positive foci in rat livers are shown in Table 4. The numbers of GST-P-positive foci in the groups administered 0.01 ppm to 1 ppm MeIQx and in the groups administered 0.0001 to 0.01 ppm DEN did not differ from the non-treatment control group. Furthermore, the numbers of GST-P-positive foci in the groups administered combinations of subcarcinogenic doses of MeIQx and DEN (0.01 ppm MeIQx + 0.0001 ppm DEN, 0.1 ppm MeIQx + 0.001 ppm DEN and 1 ppm MeIQx + 0.01 ppm DEN groups) did not differ from either their respective single treatment groups or the non-treatment control group.

In the MeIQx- and DEN-alone treatment groups, significant increases in the numbers of GST-P-positive foci were observed in the groups administered 10 ppm and 100 ppm MeIQx and in the groups administered 0.1 ppm and 1 ppm DEN. However, there was no significant difference in the number of GST-P-positive foci between the 10 ppm MeIQx + 0.1 ppm DEN group and the 10 ppm MeIQx-alone or 0.1 ppm DEN-alone groups, indicating that the hepatocar-

Table 4. GST-P-positive Foci in the Rat Liver (Experiment 2)

Group	MeIQx (ppm)	DEN (ppm)	No. of rats	GST-P-positive foci (no./cm2)
1	-	—	30	0.20 ± 0.32
2	0.01	—	30	0.17 ± 0.27
3	0.1	—	30	0.24 ± 0.30
4	1	—	30	0.21 ± 0.28
5	10	—	20	0.57 ± 0.51 [a]
6	100	—	10	13.76 ± 8.78 [a]
7	—	0.0001	30	0.30 ± 0.40
8	—	0.001	30	0.29 ± 0.39
9	—	0.01	30	0.35 ± 0.48
10	—	0.1	20	0.82 ± 0.77 [a]
11	—	1	10	8.28 ± 4.27 [a]
12	0.01	0.0001	30	0.36 ± 0.48
13	0.1	0.001	30	0.17 ± 0.24
14	1	0.01	30	0.34 ± 0.51
15	10	0.1	20	0.78 ± 0.70 [a]
16	100	1	10	33.81 ± 17.46 [a,b,c]

—, 0 ppm in the diet or drinking water. [a] Significantly different from group 1. [b] Significantly different from the 100 ppm MeIQx-alone group. [c] Significantly different from the 1 ppm DEN-alone group.

cinogenicity of a combination of low carcinogenic doses of these two carcinogens was not enhanced additively or synergistically. In contrast, the number of GST-P-positive foci in the high carcinogenic dose combination group, the 100 ppm MeIQx + 1 ppm DEN group, was synergistically increased compared with the single treatment groups.

Discussion

The present study demonstrates that concurrent treatment with subcarcinogenic doses of DEN did not enhance MeIQx-induced rat hepatocarcinogenesis. We also found that concurrent treatment with combinations of subcarcinogenic doses of DEN and MeIQx were not hepatocarcinogenic, indicating that the combined effects of subcarcinogenic doses of DEN and MeIQx were neither additive nor synergistic. Moreover, concurrent treatment with low carcinogenic doses of DEN and MeIQx did not show either additive or synergistic effects. Only in rats coadministered higher carcinogenic doses of DEN and MeIQx were synergistic effects seen. The present study provides the first experimental data on the carcinogenic effects of concurrent exposure to subcarcinogenic doses of genotoxic carcinogens belonging to different structural categories. The findings of this study are in line with previous studies on the carcinogenic effects of mixtures of chemicals[7], and provide new evidence supporting our idea that no effect levels for genotoxic carcinogens exist[1,2]. Taking into account the accumulating evidence suggesting the existence of no effect levels for genotoxic carcinogens, it is reasonable to assume that the dose-response curves of genotoxic carcinogens do not reach zero in a physiologically meaningful manner, and that threshold, at least practical threshold, doses exist for the carcinogenicity of genotoxic carcinogens.

Although several threshold mechanisms for genotoxic

carcinogens have been suggested, including induction of detoxification processes, cell cycle delay, DNA repair, and apoptosis and the suppression of neoplastically transformed cells by the immune system[3,17–19], the exact mechanisms are anticipated to be complicated and remain to be elucidated. This is especially true for combinations of multiple genotoxic carcinogens. We recently found that induction of p21[Cip/WAF1] and DNA repair enzymes such as AP endonuclease-1 (APE1) and GADD45 are at least partially responsible for the observed noncarcinogenic effect of low doses of 2-amino-3-methylimidazo[4,5-*f*]quinolone, a genotoxic heterocyclic amine, in rat livers[2]. Further studies are needed to clarify whether similar mechanisms contribute to the noncarcinogenic effect of low doses of MeIQx and/or DEN by evaluating the expression of genes involved in cell proliferation, DNA repair, metabolic activation and apoptosis.

Although combinations of carcinogens are generally considered to act in an additive or synergistic way with respect to cancer risk, recently it has been shown that carcinogens may interact in a non-syncarcinogenic way and, indeed, sometimes interact in an anticarcinogenic way, especially if they do not have a similar mode of carcinogenic action[7,20,21]. As reviewed by Ruediger H[21], interactive mechanisms by which one carcinogen may antagonize or attenuate the carcinogenic action of another carcinogen include inhibition of metabolic activation of procarcinogens, induction of metabolic inactivation, slowing down of the cell cycle via the p53 pathway, interference with the generation of DNA-adducts, and induction of apoptosis. The fact that concurrent treatment with low carcinogenic doses of MeIQx and DEN did not show additive or synergistic effects while combinations of the 2 carcinogens showed synergistic effects at higher carcinogenic doses suggests that combinations of MeIQx and DEN do not exert carcinogenicity in a simple dose-dependent manner and also indicates the possibility of antagonistic interaction between these 2 carcinogens when administered at low doses. Further research to clarify the interactive mechanisms mentioned above will not only facilitate the understanding of the carcinogenicity of combinations of MeIQx and DEN, but will also expand the understanding of threshold mechanisms.

In conclusion, the present study demonstrated that concurrent treatment with noncarcinogenic doses of DEN and MeIQx did not exert hepatocarcinogenicity in rats and that their combined effects were neither additive nor synergistic. These findings further support our idea that there is a threshold, at least a practical threshold, that should be considered when evaluating the risk of genotoxic carcinogens. Dose-response relationships for low dose genotoxic carcinogens, especially for mixtures of carcinogens, are still controversial in the field of carcinogen risk assessment. Further accumulation of data, especially mechanistic data, should be promoted to facilitate not only our understanding of the carcinogenic effects of low doses of genotoxic carcinogens, but also to establish accurate means of risk assessment.

References

1. Fukushima S, Wei M, Kakehashi A, and Wanibuchi H. Cancer Risk Assessment, Thresholds for Genotoxic Carcinogens: Evidence from Mechanism –Based Carcinogenicity Studies. John Wiley and Sons, Inc., 2010.

2. Wei M, Wanibuchi H, Nakae D, Tsuda H, Takahashi S, Hirose M, Totsuka Y, Tatematsu M, and Fukushima S. Low-dose carcinogenicity of 2-amino-3-methylimidazo[4,5-f] quinoline in rats: Evidence for the existence of no-effect levels and a mechanism involving p21(Cip/WAF1). Cancer Sci. **102**: 88–94. 2011. [Medline] [CrossRef]

3. Sofuni T, Hayashi M, Nohmi T, Matsuoka A, Yamada M, and Kamata E. Semi-quantitative evaluation of genotoxic activity of chemical substances and evidence for a biological threshold of genotoxic activity. Mutat Res. **464**: 97–104. 2000. [Medline]

4. Williams GM, Iatropoulos MJ, and Jeffrey AM. Mechanistic basis for nonlinearities and thresholds in rat liver carcinogenesis by the DNA-reactive carcinogens 2-acetylaminofluorene and diethylnitrosamine. Toxicologic pathology. **28**: 388–395. 2000. [Medline] [CrossRef]

5. Waddell WJ, Fukushima S, and Williams GM. Concordance of thresholds for carcinogenicity of N-nitrosodiethylamine. Archives of toxicology. **80**: 305–309. 2006. [Medline] [CrossRef]

6. Waddell WJ. Thresholds of carcinogenicity in the ED01 study. Toxicological Sciences. **72**: 158–163. 2003. [Medline] [CrossRef]

7. Shirai T, Ogawa K, and Takahashi S. Carcinogenic Effects of mixtures of chemicals. J Toxicol Pathol. **19**: 1–13. 2006. [CrossRef]

8. Berger MR, Schmahl D, and Zerban H. Combination experiments with very low doses of three genotoxic N-nitrosamines with similar organotropic carcinogenicity in rats. Carcinogenesis. **8**: 1635–1643. 1987. [Medline] [CrossRef]

9. Hasegawa R, Shirai T, Hakoi K, Takaba K, Iwasaki S, Hoshiya T, Ito N, Nagao M, and Sugimura T. Synergistic enhancement of glutathione S-transferase placental form-positive hepatic foci development in diethylnitrosamine-treated rats by combined administration of five heterocyclic amines at low doses. Jpn J Cancer Res. **82**: 1378–1384. 1991. [Medline] [CrossRef]

10. Ito N, Hasegawa R, Shirai T, Fukushima S, Hakoi K, Takaba K, Iwasaki S, Wakabayashi K, Nagao M, and Sugimura T. Enhancement of GST-P positive liver cell foci development by combined treatment of rats with five heterocyclic amines at low doses. Carcinogenesis. **12**: 767–772. 1991.

11. Hasegawa R, Kato T, Hirose M, Takahashi S, Shirai T, and Ito N. Enhancement of hepatocarcinogenesis by combined administration of food-derived heterocyclic amines at low doses in the rat. Food Chem Toxicol. **34**: 1097–1101. 1996. [Medline] [CrossRef]

12. Fukushima S, Wanibuchi H, Morimura K, Wei M, Nakae D, Konishi Y, Tsuda H, Uehara N, Imaida K, Shirai T, Tatematsu M, Tsukamoto T, Hirose M, Furukawa F, Wakabayashi K, and Totsuka Y. Lack of a dose-response relationship for carcinogenicity in the rat liver with low doses of 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline or N-nitrosodiethylamine. Jpn J Cancer Res. **93**: 1076–1082. 2002. [Medline] [CrossRef]

13. Tsuda H, Fukushima S, Wanibuchi H, Morimura K, Nakae D, Imaida K, Tatematsu M, Hirose M, Wakabayashi K, and Moore MA. Value of GST-P positive preneoplastic hepatic foci in dose-response studies of hepatocarcinogenesis: evidence for practical thresholds with both genotoxic and non-genotoxic carcinogens. A review of recent work. Toxicol Pathol. **31**: 80–86. 2003. [Medline]

14. Wei M, Hori TA, Ichihara T, Wanibuchi H, Morimura K, Kang JS, Puatanachokchai R, and Fukushima S. Existence of no-observed effect levels for 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline on hepatic preneoplastic lesion development in BN rats. Cancer Lett. **231**: 304–308. 2006. [Medline] [CrossRef]

15. Fukushima S, Kinoshita A, Puatanachokchai R, Kushida M, Wanibuchi H, and Morimura K. Hormesis and dose-response-mediated mechanisms in carcinogenesis: evidence for a threshold in carcinogenicity of non-genotoxic carcinogens. Carcinogenesis. **26**: 1835–1845. 2005. [Medline] [CrossRef]

16. Wei M, Hamoud AS, Yamaguchi T, Kakehashi A, Morimura K, Doi K, Kushida M, Kitano M, Wanibuchi H, and Fukushima S. Potassium bromate enhances N-ethyl-N-hydroxyethylnitrosamine-induced kidney carcinogenesis only at high doses in Wistar rats: indication of the existence of an enhancement threshold. Toxicol Pathol. **37**: 983–991. 2009. [Medline] [CrossRef]

17. De Flora S. Threshold mechanisms and site specificity in chromium(VI) carcinogenesis. Carcinogenesis. **21**: 533–541. 2000. [Medline] [CrossRef]

18. Lutz WK, and Kopp-Schneider A. Threshold dose response for tumor induction by genotoxic carcinogens modeled via cell-cycle delay. Toxicol Sci. **49**: 110–115. 1999. [Medline] [CrossRef]

19. Kirsch-Volders M, Aardema M, and Elhajouji A. Concepts of threshold in mutagenesis and carcinogenesis. Mutat Res. **464**: 3–11. 2000. [Medline]

20. United States Environmental Protection Agency Supplementary Guidance for Conducting Health Risk Assessment of Chemical Mixtures (2000). http://cfpub.epa.gov/ncea/cfm/recordisplay.cfm?deid=20533.

21. Ruediger HW. Antagonistic combinations of occupational carcinogens. International Archives of Occupational and Environmental Health. **79**: 343–348. 2006. [Medline] [CrossRef]