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DNA Adducts Formed by 2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline in Rat Liver: Dose-Response on Chronic Administration

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The effect of administration of 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) at various doses on DNA adduct formation in male rats was examined by ^{32}P-postlabeling analysis. Administration of MeIQx in the diet at 0.4 ppm, 4 ppm, 40 ppm and 400 ppm for one week resulted in the formations of 0.04, 0.28, 3.34 and 39.0 adducts per 10^7 nucleotides in rat liver cells. Continuous administration of 400 ppm of MeIQx in the diet for 61 weeks to rats induced hepatocellular carcinomas in all rats. The carcinogenicity of MeIQx at doses of 40 ppm or less is not known yet, but the above results show a linear relationship between the level of MeIQx administered and the adduct level. In rats treated with low doses of 0.4, 4 and 40 ppm of MeIQx, adduct levels increased linearly with time of treatment, the levels in week 12 being two to three times those in week 1. In contrast, on treatment with 400 ppm of MeIQx, the adduct level in the liver increased until week 4, when it was 110 adducts per 10^7 nucleotides, and then remained constant for the next 8 weeks. Induction of the multidrug-resistance gene was suggested to be involved in development of this plateau level.

Key words: DNA adducts — ^{32}P-Postlabeling analysis — MeIQx — *mdr* gene

Heterocyclic amines present in cooked foods are highly mutagenic to *Salmonella* on metabolic activation and are carcinogenic to rodents.[1,2] Heterocyclic amines can be metabolized to mutagenic derivatives by liver S9 of rodents and monkeys and also by that of humans.[2-4] We showed that one of these heterocyclic amines, 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ),[6] formed DNA adducts in both hepatic and extrahepatic tissues of monkeys.[5] Furthermore, IQ was recently found to be carcinogenic in monkeys.[6] These results suggest that heterocyclic amines may play some roles as etiological agents in the development of cancer in humans. Although humans consume heterocyclic amines in food continuously throughout life, the amount present in ordinary cooked food is very small, being about 0.1–70 ng/g.[7-9] However, in assessing the carcinogenic risks of chemicals to human, even the biological effects of chemicals at very low doses must be taken into consideration.

In the present work, we examined DNA adduct formation in the liver of rats treated with doses of 0.4–400 ppm of carcinogen. As a model carcinogen, we used 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) because it is strongly carcinogenic in rodents, inducing hepatocellular carcinomas, Zymbal gland squamous cell carcinomas, etc. in rats[10] and tumors of the liver, lung, etc. in mice,[11] and because it is present in cooked foods.[8,9] To detect the low levels of DNA adducts expected from the low doses of MeIQx administered, we used the ^{32}P-postlabeling method.[12,13] We also examined expression of the multidrug-resistance (*mdr*) gene and induction of foci positive for glutathione *S*-transferase of the placental form (GST-P), which are markers of preneoplastic lesions in rat liver,[14,15] after treatment with various doses of MeIQx.

MATERIALS AND METHODS

Chemicals MeIQx was obtained from Nard Institute, Osaka. The purity of MeIQx was determined to be more than 99% by elemental analysis, HPLC and NMR, IR and UV spectrometries. Polyethyleneimine-cellulose sheets and the enzymes used for ^{32}P-postlabeling were obtained as described previously.[5,16] The reagents used for northern blotting were as described elsewhere.[17]

Animals Male Fischer 344 rats (6 weeks old) were purchased from Charles River Japan Inc., Kanagawa. MeIQx was added at concentrations of 0.4, 4, 40 and 400 ppm to the powder diet (CE-2, CLEA Japan, Tokyo), and the mixtures were pelleted at room temperature and dried at 70°C for 3 h. The rats were given pellet diet

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[6] Abbreviations: MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]-quinoxaline; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; *mdr*, multidrug-resistance; GST-P, placental type of glutathione *S*-transferase.

containing MeIQx for the periods indicated in the text. MeIQx in the diet of each group was determined by HPLC after extraction with 50% methanol. More than 90% of the added MeIQx was found to be present in the diet of each group. Control rats received basal pellet diet (CE-2). All measurements were made on two rats. Food and water were provided *ad libitum*. Rats were anesthetized with diethyl ether and decapitated and the liver and other organs were promptly excised, frozen and stored at −80°C until DNA isolation.

^{32}P-Postlabeling of MeIQx-DNA adducts DNA was isolated by phenol extraction. Digestion of DNA and ^{32}P-labeling of the digest were carried out as reported previously.[5,16] Briefly, 0.17 μg of digested DNA was ^{32}P-labeled with 45 μM (600 Ci/mmol) of [γ-^{32}P]ATP under standard conditions and 5 μg of digested DNA was ^{32}P-labeled with 3.5 μM (6000 Ci/mmol) of [γ-^{32}P]ATP under adduct-intensification (ATP-deficient) conditions. The separation of DNA adducts on polyethyleneimine-cellulose thin layer chromatographic sheets, detection of adducts by autoradiography and quantitation of adducts were carried out by the methods used in studies on IQ-DNA adducts.[5]

Isolation of RNA and northern blotting RNA was obtained from frozen liver by the guanidinium/hot phenol procedure as described[18] and poly(A)$^+$RNA was

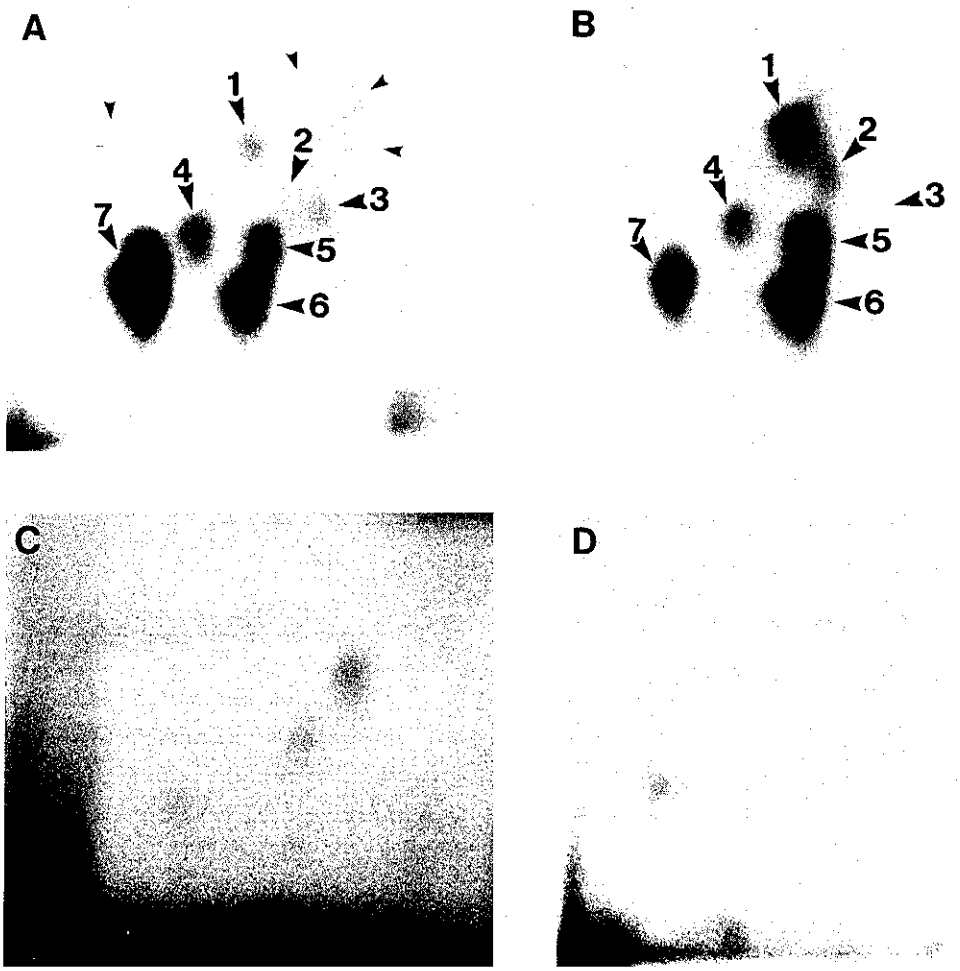

Fig. 1. MeIQx-DNA adducts revealed by ^{32}P-postlabeling in the liver of rats treated with 400 ppm of MeIQx for 1 week. Adducts were analyzed under standard conditions (A, MeIQx; C, control) and adduct-intensification conditions (B, MeIQx; D, control). Development was done from bottom to top in 2.5 *M* lithium formate, 4.7 *M* urea, pH 3.5, and from left to right in 0.8 *M* LiCl, 0.4 *M* Tris-HCl, 6.8 *M* urea, pH 8.0. X-Ray films were exposed for 12 h (standard, MeIQx), 2 days (standard, control), 1 h (intensification, MeIQx) and 24 h (intensification, control) at −70°C.

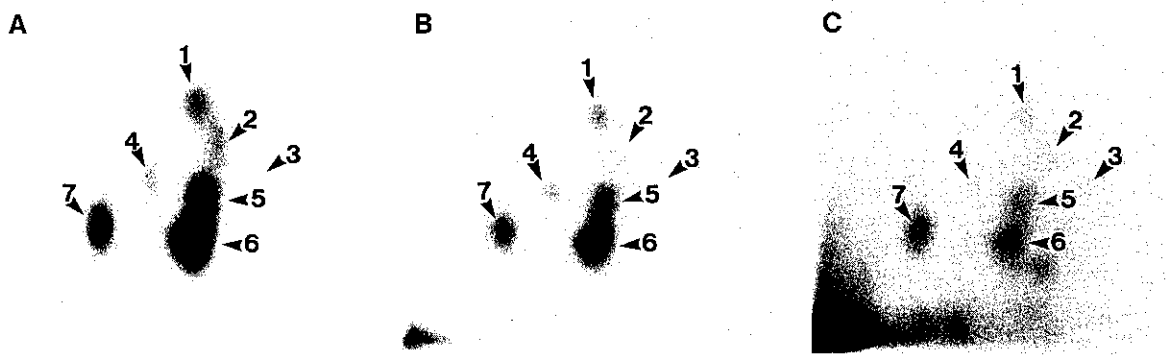

Fig. 2. MeIQx-DNA adducts in the liver of rats treated with 40 (A), 4 (B) or 0.4 (C) ppm of MeIQx for 1 week. Adducts were analyzed under adduct-intensification conditions and developed as described for Fig. 1. X-Ray films were exposed for 6 h (40 ppm), 24 h (4 ppm) or 48 h (0.4 ppm) at −70°C.

Table I. Adduct Levels of Each Spot in Rat Liver DNA after Administration of MeIQx for 1 Week

Spot No.	Adduct level[a]: adducts per 10^7 nucleotides			
	0.4 ppm[b]	4 ppm[b]	40 ppm[b]	400 ppm[c]
1	0.0020	0.012	0.15	2.0
2	0.0018	0.012	0.12	1.5
3	0.0015	0.024	0.18	1.9
4	0.0038	0.018	0.19	3.3
5	0.0054	0.045	0.34	4.6
6	0.0062	0.055	0.71	10.0
7	0.0192	0.115	1.67	15.7
Total[d]	0.0399	0.281	3.36	39.0

a) The reported DNA adduct levels are the average of four assays, two each from two rats.
b) ^{32}P-Postlabeling assay was carried out under adduct-intensification conditions and adduct levels were determined from intensification factors obtained by measurements on samples from the 400 ppm group. Typical intensification factors of spots under the conditions used were as follows: spot 1, 423; spot 2, 303; spot 3, 59.5; spot 4, 239; spot 5, 309; spot 6, 319; spot 7, 75.2.
c) ^{32}P-Postlabeling assay was carried out under the standard conditions.
d) The total adduct levels per 10^7 nucleotides in two rats were 0.0346 and 0.0418 for 0.4 ppm, 0.148 and 0.411 for 4 ppm, 2.18 and 4.50 for 40 ppm, 36.4 and 41.6 for 400 ppm.

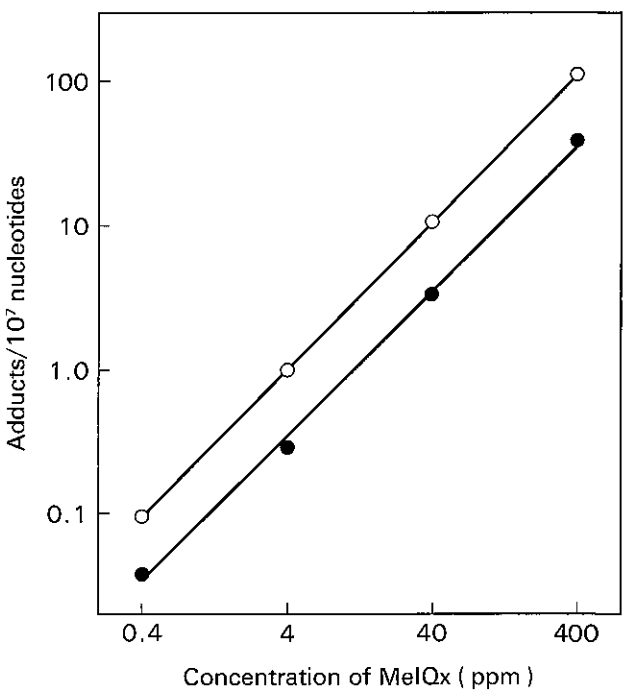

Fig. 3. Dose-response relationship of total adduct level of MeIQx-DNA adducts in rat liver in week 1 (●) and week 12 (○). Adduct levels in 400 ppm-treated samples were measured under the standard conditions and those in other samples under adduct-intensification conditions. Values were determined as described in Table I.

isolated by oligo(dT)-cellulose chromatography.[17] RNA was separated, transferred to nitrocellulose sheets and hybridized with the 0.8 kb *Pvu*II fragment of pHDR 4.4, a cDNA of the human *mdr*1 gene[19] provided by Dr. I. B. Roninson, University of Illinois College of Medicine.

Detection of GST-P-positive foci Paraffin sections were prepared from liver slices fixed in 10% neutralized formalin, and cut and stained immunohistochemically with specific antibodies to GST-P.[14,15] The antibodies to GST-P were provided by Dr. K. Sato, Hirosaki University.

RESULTS

Body weights of rats in all groups increased time-dependently. The average body weights of rats fed diets containing 0.4, 4 and 40 ppm MeIQx did not differ from those of control rats, whereas those of rats given the diet containing 400 ppm MeIQx were 10% less than those of control rats throughout the experimental period of 12 weeks. The average intakes of MeIQx in rats given 0.4, 4, 40 and 400 ppm MeIQx were 6.5, 65.2, 672 and 6280 μg/day/rat, respectively.

Fig. 1 shows autoradiograms of MeIQx-DNA adducts in the liver of rats given 400 ppm of MeIQx in their diet for 1 week. Under the standard conditions, 11 spots could be detected (spots 1 to 7 and 4 other spots indicated by small arrowheads without numbers). The 4 spots without numbers were not detected under the adduct-intensification conditions. Adduct-intensification conditions had to be applied for detection and quantitation of adducts formed in groups given lower doses than 400 ppm. Thus, spots 1 to 7 were quantitated in the samples analyzed under both standard and adduct-intensification conditions. Autoradiograms of samples from rats given 40 ppm, 4 ppm and 0.4 ppm of MeIQx for 1 week are presented in Fig. 2. In all cases, the same 7 spots as those in samples from rats given 400 ppm of MeIQx were detected by labeling under adduct-intensification conditions.

The adduct levels after administration of each level of MeIQx for week 1 are shown in Table I. In all cases, spot 7 was the major adduct, constituting 40–50% of the total. The levels of both total adducts and adducts in each spot increased dose-dependently. Fig. 3 shows the linear dose-response relationships between the level of MeIQx administered and adduct formation in week 1 and week 12.

We next examined the effect of the exposure time on the formation of DNA adducts. As shown in Fig. 4, the adduct levels in the groups given 0.4, 4 and 40 ppm of MeIQx increased with the time of treatment for at least 12 weeks. The rates of increase in adduct levels were the same in the groups given 0.4, 4 and 40 ppm of MeIQx. However, in the 400 ppm group, the rate of increase was higher than in other groups until week 4 and then the level remained constant until week 12. Food consump-

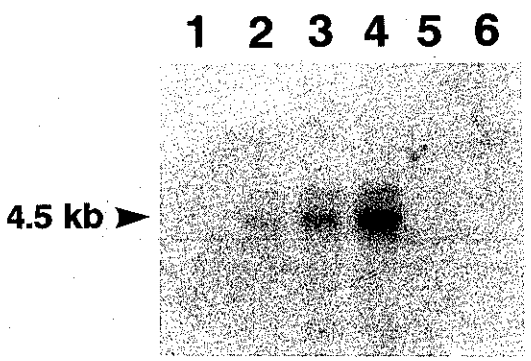

Fig. 5. Expression of multidrug-resistance gene in the liver of rats treated with MeIQx. 1, 400 ppm MeIQx for 1 week; 2, 400 ppm MeIQx for 4 weeks; 3, 400 ppm MeIQx for 8 weeks; 4, 400 ppm MeIQx for 12 weeks; 5, basal diet for 12 weeks; 6, 40 ppm MeIQx for 12 weeks. Samples of 5 μg of poly(A)$^+$RNA were analyzed. The probe was hybridized at 42°C in the solution reported[17] and the filter was washed with 2×SSC at 42°C. X-Ray films were exposed for 72 h.

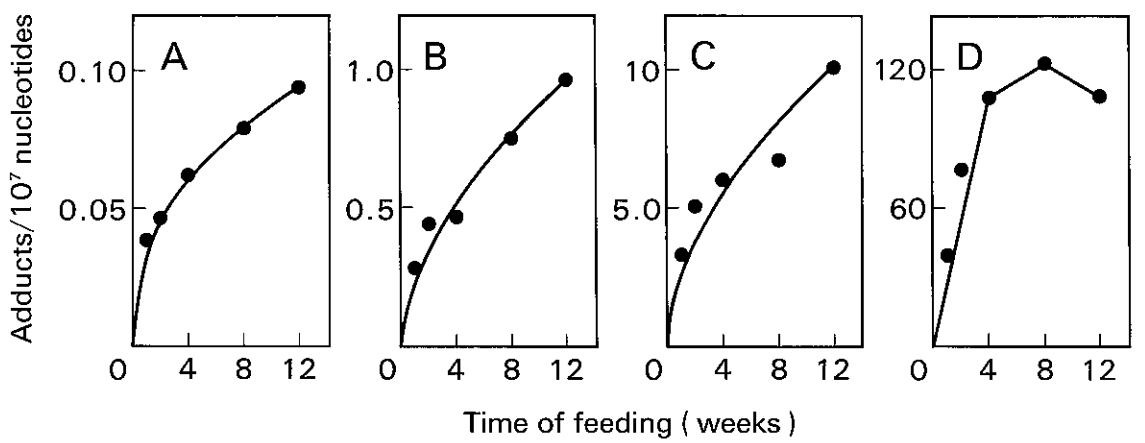

Fig. 4. Change of DNA adduct levels in the liver of rats treated with 0.4 (A), 4 (B), 40 (C) or 400 (D) ppm of MeIQx for 12 weeks. Adduct levels were measured as described in Table I.

tion in this group from week 6 to week 12 was almost invariable.

In the 400 ppm group, the *mdr* gene was expressed when the adduct level became constant after week 4 (Fig. 5). Expression of the *mdr* gene was higher in week 8 and week 12 than in week 4. No expression of the *mdr* gene was detected in the livers of rats given 0.4, 4 and 40 ppm of MeIQx in the diet for 12 weeks. Enzyme-altered foci expressing GST-P were observed in the liver of the group given 400 ppm of MeIQx in the diet for 8 and 12 weeks, but not 1 or 4 weeks. Areas of GST-P-positive lesions increased with the period of treatment, and in weeks 8 and 12, the average areas of GST-P positive lesions were 0.18 and 4.0 mm^2/cm^2, respectively. In the group given 400 ppm of MeIQx, whitish foci were detected macroscopically in the liver in week 8 and their number was greater in week 12. No GST-P-positive foci were observed in other groups.

DISCUSSION

In this study, we showed that MeIQx, a representative food carcinogenic heterocyclic amine, formed DNA adducts even when administered at 0.4 ppm in the diet, which is 1000 times less than the dose used in carcinogenesis experiments.[10, 11] We also found that the level of adducts increased with the time of MeIQx treatment. These results, together with the finding of a linear relationship between the dose and the level of adducts, suggest that even lower doses of carcinogens would result in formation of DNA adducts, probably without a threshold, and that these adducts would accumulate to some extent. The detection limit of the ^{32}P-postlabeling method is one per 10^9 nucleotides. Adducts of MeIQx with DNA could probably be detected in DNA samples from humans, who are usually continuously exposed to heterocyclic amines including MeIQx.

In the group treated with 400 ppm of MeIQx, DNA adducts in the liver accumulated at higher rates time- and dose-dependently than in other groups until week 4. This rapid accumulation may be explained by the induction of metabolic enzymes, such as the cytochrome P450 monooxygenase system.[20] A single dose of MeIQx (0.22 mmol/kg) in rats was recently found to induce the cytochrome P-448H isozyme, which metabolizes MeIQx.[21] DNA adducts in the kidney, colon and brain of rats given 400 ppm of MeIQx in the diet were also analyzed. All DNA adducts observed in the liver were also formed in these extrahepatic tissues and spots 6 and 7 were the major adducts in the kidney, colon and brain. As shown in Fig. 6, the total adduct levels in the kidney were in the same order as those in the liver, but the time courses of their accumulation were different: the level of adducts increased with increase in the period of feeding in the kidney, unlike in the liver. Much lower levels of adducts were formed in the colon and brain, and the levels reached maxima in week 4 and then became constant or decreased. The plateau or maximal levels were different in different organs.

Expression of the *mdr* gene was observed in the group treated with 400 ppm of MeIQx for 4 weeks. The initiated cells are reported to be resistant to toxic damage by chemicals.[22] Some of this resistance is attributable to the function of P-glycoprotein, a product of the *mdr* gene, in excluding chemicals from cells.[23–25] Phase II detoxifying enzymes such as GST-P are also reported to be expressed in initiated cells.[14, 25] We observed cells expressing GST-P by immunocytochemistry in week 4. However, glutathione conjugates of heterocyclic amine compounds such as IQ, have so far not been observed (unpublished

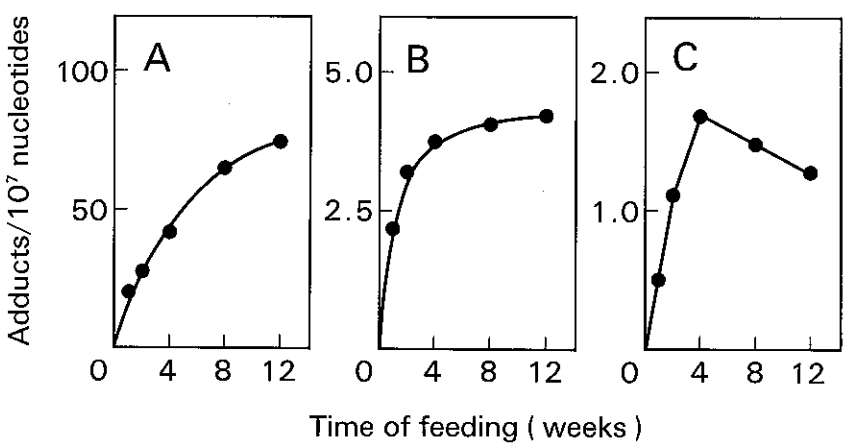

Fig. 6. Time courses of change in MeIQx-DNA adduct levels in the kidney (A), colon (B) and brain (C) of rats treated with 400 ppm of MeIQx for 12 weeks. The adducts in the kidney were analyzed under standard conditions, and those in the colon and brain under adduct-intensification conditions.

data), although no data are available about the formation of a MeIQx-glutathione conjugate.

For initiation, the formation of DNA adducts is a prerequisite and the DNA damage must then be fixed as mutations. Moreover, for tumor production, multiple genetic changes should be necessary.[26, 27] The significance of DNA adducts formed by administration of low doses of carcinogens should be further investigated, because in daily life humans are constantly exposed to small amounts of various carcinogens. Furthermore, the effects of promoters to which humans may be exposed also remain to be clarified.

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