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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

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Potential synergism among 5 heterocyclic amines at low doses in the induction of glutathione *S*-transferase placental form (GST-P)-positive liver cell foci was examined in an 8-week experiment using male rats initially given diethylnitrosamine (200 mg/kg, ip). The heterocyclic amines applied were 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (500 ppm), 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]-imidazole (500 ppm), 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole (800 ppm), 2-amino-9*H*-pyrido[2,3-*b*]indole (800 ppm), and 2-amino-1-methyl-6-phenylimidazo[4, 5-*b*]pyridine (PhIP, 400 ppm). Separate groups received each chemical at the dose used in earlier carcinogenicity assays (above doses), at 1/5 or 1/25 of these, or all 5 chemicals together, each at the 1/5 or 1/25 levels. The numbers and areas of GST-P-positive foci were significantly increased with all chemicals, except for PhIP, at the highest dose, the results being consistent with the reported liver carcinogenicity. In the combined treatment at the 1/5 dose levels, synergistic enhancement occurred; the numbers and areas of foci were significantly increased above the sums of individual data. However, this was not the case for the 1/25 dose groups. Although the synergism between pyrolysis products in liver carcinogenesis depended on the dose and combination of chemicals, the findings, together with those from a previous experiment using 5 different heterocyclic amines, are of particular significance since several heterocyclic amines might be simultaneously generated during cooking of foodstuffs.

Key words: Hepatocarcinogenesis — GST-P — Heterocyclic amine — Low-dose combination — F344 rat

Most human cancers may be caused by exposure of individuals to environmental carcinogenic agents,[1–3] dietary habits in particular being regarded as main determinants for genesis of cancer in man.[4–7] In recent years, a number of heterocyclic amines from charred parts of broiled fish and meat have been demonstrated to exert high mutagenicity toward *Salmonella typhimurium* TA 98 and TA 100 strains[8–11] and also to possess tumorigenic potential in either mice or rats.[12–14] Although the doses used in the animal carcinogenicity studies were relatively high as compared to the amounts ordinarily consumed in daily life, the fact that carcinogenic substances are actually produced in cooked food is clearly extremely important.

Under normal environmental conditions, humans are exposed concomitantly or sequentially to several carcinogens at low doses. Syncarcinogenesis is therefore likely to be an important problem. Synergistic effects of chemicals in experimental carcinogenesis have been demonstrated in many organs[15–19] and using a medium-term bioassay system of 8 weeks' duration developed in our laboratory,[20, 21] we previously demonstrated additive or synergistic effects of various liver carcinogens and heterocyclic amines.[22, 23] The experimental system has clear advantages for investigation of combined effects where a large number of groups is necessary.

In the present study, the influence of combined treatment with 5 carcinogenic heterocyclic amines at low dose levels was further investigated using the medium-term DEN-PH bioassay method. The five heterocyclic amines chosen were 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2[5]),[12, 24] 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1),[25, 26] 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole (MeAαC),[26, 27] 2-amino-9*H*-pyrido-[2,3-*b*]indole (AαC),[26, 27] and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP).[28, 29] All these compounds, except for PhIP, are carcinogenic to the liver in rats and/or mice. Doses selected for the present combination study were 1/5 and 1/25 of the reported carcinogenic doses (Glu-P-1, MeAαC, AαC, and PhIP)

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[5] Abbreviations used are: Trp-P-2, 3-amino-1-methyl-5*H*-pyrido-[4,3-*b*]indole; Glu-P-1, 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole; MeAαC, 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]-indole; AαC, 2-amino-9*H*-pyrido[2,3-*b*]indole; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; DEN, diethylnitrosamine; GST-P, placental form of glutathione *S*-transferase; PH, 2/3 partial hepatectomy.

or the re-evaluated dose based on the toxicity observed in previous experiments (Trp-P-2).[24]

MATERIALS AND METHODS

Animals and chemicals A total of 447 male F344 rats, 5 weeks old, were obtained from Charles River Japan Inc., Atsugi. They were housed, 5 rats per cage, with wood-chip bedding in an air-conditioned animal room at 23±2°C and 55±5% humidity. Food (Oriental MF, Oriental Yeast Co., Tokyo) and water were available *ad libitum* throughout the experiment. Diethylnitrosamine (DEN) was obtained from Tokyo Chemical Industry Co., Tokyo. Trp-P-2 (Trp-P-2 acetate), MeAαC (MeAαC acetate), AαC (AαC acetate), and PhIP (PhIP hydrochloride) were obtained from the Nard Institute, Osaka, and Glu-P-1 (Glu-P-1 hydrochloride) was from Katsura Chemical Co., Tokyo. The purities of these heterocyclic amines were confirmed by high-performance liquid chromatography, mass spectrometry, infrared spectrophotometry, and elemental analysis. The chemicals were incorporated into powdered basal diet (Oriental MF) using a mixer without adding oil and stored in a cold room until use.

Treatment of animals The experimental protocol is shown in Fig. 1. After a 1 week initial observation period, the rats in group 1 (15–18 rats in each chemical treatment subgroup) were intraperitoneally injected with DEN dissolved in 0.9% NaCl at a dose of 200 mg/kg body weight. Starting 2 weeks later, the animals were maintained on powdered diet supplemented with heterocyclic amine(s) as listed below. Group 2 (16 rats) was given DEN in the same manner as for group 1 without subsequent administration of any test compound. Group 3 rats (10 or 11 rats in each chemical treatment subgroup) were injected with 0.9% NaCl instead of DEN solution and then administered test compound(s) for 6 weeks. All rats were subjected to two-thirds partial hepatectomy (PH) at the end of week 3.

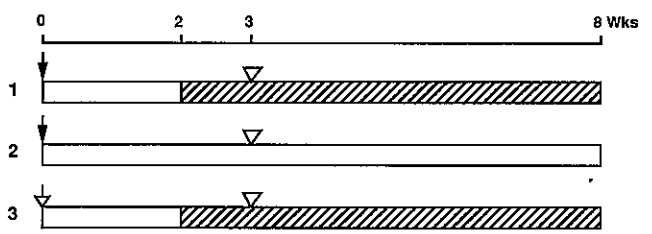

Fig. 1. Experimental design. As test chemicals, 5 heterocyclic amines were administered individually or in combination. The chemicals were incorporated into powdered basal diet at 20, 100, and 500 ppm for Trp-P-2 and Glu-P-1, 32, 160, and 800 ppm for MeAαC and AαC and 16, 80, 400 ppm for PhIP. The highest dose levels were the same as used previously in carcinogenicity studies (carcinogenic dose), except for Trp-P-2, and others were 1/5 and 1/25 of the highest dose. Two subgroups were administered all 5 chemicals together at the 1/5 or 1/25 doses. Immediately upon killing at week 8, 3 liver slices per animal were fixed in ice-cold acetone and processed for immunohistochemical demonstration of GST-P, an enzyme marker for preneoplastic lesions. ↓: diethylnitrosamine, 200 mg/kg i.p. ↓: saline, 4 ml/kg i.p. ▽: 2/3 partial hepatectomy. ▨: heterocyclic amine(s) in diet.

Three dose levels were used for each heterocyclic amine, the highest being the same as the dose used in long-term studies (possible carcinogenic dose) or the re-evaluated dose for Trp-P-2, the others being 1/5 and 1/25 of this. The highest doses used were 500 ppm for Glu-P-1, 800 ppm for MeAαC, and AαC, and 400 ppm for PhIP. Two subgroups in groups 1 and 3 were given all 5 chemicals in combination at the 1/5 or 1/25 levels for examination of additive or synergistic effects in carcinogenic potential. For Trp-P-2, the highest dose was set at 500 ppm since no toxicity was observed in the carcinogenicity study, in which the dose was 100 ppm.[24]

Food consumption was measured during the period of test chemical feeding and body weights were recorded every 2 weeks. All animals were killed at week 8, and immediately thereafter, the livers were excised and cut into 2–3 mm thick slices, three of which, one from the caudate lobe and two from the right anterior lobe, were fixed in ice-cold acetone solution for immunohistochemical examination of GST-P expression. GST-P immunohistochemistry was performed as previously described.[30] The numbers and the areas of GST-P-positive foci of more than 0.1 mm in diameter and the total areas of the liver sections examined were measured using a video image processor (VIP-21C, Olympus Co., Tokyo). The results were assessed by comparing the values of foci between group 1 (DEN-test compounds) and group 2 (DEN alone). Group 3 animals served to assay the potential of the test chemicals to induce GST-P-positive foci without prior DEN.

Statistical analysis Statistical analysis of differences between means was carried out using Student's t test and Welch's t test in combination with the F test. To determine whether the combined treatments acted additively or synergistically, linear statistical inference was employed.[31] The value of t was calculated using the following equation:

$$t=\frac{Y_{comb}+4\times Y_0-Y_1-Y_2-Y_3-Y_4-Y_5}{\sqrt{Ve\times(1/n_{comb}+16/n_0+1/n_1+1/n_2+1/n_3+1/n_4+1/n_5)}}$$

$$df=(n_{comb}-1)+(n_0-1)+(n_1-1)+(n_2-1)+(n_3-1)+(n_4-1)+(n_5-1)$$

where Y is mean value of foci and n is number of samples in the combined treatment (comb), control (0) and each

of the single chemical treatment (1–5) groups, and Ve is the mean square error. The t value was assessed in the t table at the appropriate degree of freedom for error (df).

RESULTS

Final body and liver weights and data on food consumption during the period of chemical feeding are summarized in Table I. Body weights as compared to DEN alone group values were decreased in the Glu-P-1, MeAαC and PhIP carcinogenic dose groups and the 1/5 combined treatment group. Liver weights were increased in all treated groups, especially with MeAαC and the combined treatment. Food consumption was slightly decreased in groups given the carcinogenic doses of Glu-P-1, MeAαC and PhIP.

Numbers and areas of GST-P-positive foci per unit area of liver section after and without DEN initiation are summarized in Tables II and III, respectively. In groups given Trp-P-2, Glu-P-1, MeAαC or AαC at the doses used in carcinogenicity studies, values for both parameters were significantly increased over the control levels. With PhIP, there was no significant difference in either number or area. Glu-P-1 was the most effective enhancer, followed by MeAαC. Even at the 1/5 dose level, positive results were obtained with Glu-P-1 for both parameters and with Trp-P-2 and MeAαC for either number or area. Furthermore, Glu-P-1 and AαC showed positive results even at the 1/25 dose level. Dose-dependent increase of GST-P-positive foci induction after initiation with DEN was most clearly observed with Glu-P-1 and was also evident with MeAαC, Trp-P-2 and AαC. PhIP did not exert any dose dependency in the induction of foci. In the groups without DEN initiation, dose-dependent development of foci was observed with all chemicals except for PhIP and MeAαC; the latter induced large foci in one animal of the middle dose group.

Combined treatment of rats with 5 heterocyclic amines increased both numbers and areas of foci at the 1/5 dose levels, but did not do so at the 1/25 dose levels. The increase at the 1/5 doses over the sum of the individual data was statistically significant at $P<0.05$ for both

Table I. Food Consumption Data and Rat Body and Liver Weights

Group/ Treatment (dose level)[a]			DEN-initiated					Non-initiated (Group 3)				
			No. of rats	Final weight (g)			Food consumption (g/day/rat)[b]	No. of rats	Final weight (g)			Food consumption (g/day/rat)
				Body	Liver				Body	Liver		
					(g)	(%)				(g)	(%)	
1	Trp-P-2	(1/1)	15	259±13	9.42[c]	3.64[c]	16.2	10	284±9	9.66	3.40	15.6
	Glu-P-1	(1/1)	18	224±14[c]	7.34	3.28[c]	13.8	11	239±9	8.23	3.46	13.5
	MeAαC	(1/1)	16	237±12[c]	12.86[c]	5.42[c]	13.4	11	245±9	12.42	5.08	14.4
	AαC	(1/1)	17	259±15	8.36[e]	3.22[d]	15.4	10	280±10	9.86	3.52	16.5
	PhIP	(1/1)	15	233±11[c]	7.63	3.26[c]	13.2	10	242±14	8.26	3.41	19.5
	Trp-P-2	(1/5)	16	273±10[c]	8.89[c]	3.25[c]	15.2	11	284±14	9.20	3.24	16.5
	Glu-P-1	(1/5)	15	260±19	8.48[d]	3.25[d]	15.7	11	280±13	9.26	3.31	16.8
	MeAαC	(1/5)	15	260±15	9.01[c]	3.46[c]	16.3	11	283±12	10.07	3.55	17.7
	AαC	(1/5)	14	264±19	7.93	2.99	17.4	11	294±14	9.44	3.21	16.6
	PhIP	(1/5)	16	260±17	7.86	3.03	15.2	11	297±14	9.90	3.34	16.6
	Combined	(1/5)	15	246±14[c]	9.96[c]	4.06[c]	15.3	9	261±7	9.56	3.66	14.9
	Trp-P-2	(1/25)	15	266±22	8.24[e]	3.08	16.2	11	296±10	9.81	3.31	17.1
	Glu-P-1	(1/25)	16	263±11	7.97[e]	3.02	15.3	11	287±13	9.19	3.20	17.6
	MeAαC	(1/25)	16	261±20	7.82	2.98	16.9	10	283±14	9.02	3.19	17.7
	AαC	(1/25)	16	260±18	7.34	2.82	16.1	11	285±15	8.85	3.10	16.7
	PhIP	(1/25)	15	254±26	7.48	2.94	17.2	11	282±15	8.54	3.03	17.5
	Combined	(1/25)	17	262±19	8.26[e]	3.16[e]	15.6	11	280±17	9.75	3.50	14.7
2	None		16	261±19	7.24	2.84	16.0	—				

Data are mean±SD values.
a) Relative to the dose levels used in carcinogenicity studies or the re-evaluated dose for Trp-P-2.
b) Food consumption during the period of chemical feeding.
c) Significantly different from group 2 at $P<0.001$.
d) Significantly different from group 2 at $P<0.01$.
e) Significantly different from group 2 at $P<0.05$.

Table II. Numbers and Areas of GST-P-positive Foci in the Livers of Rats Initiated with DEN

	Group/Treatment (dose)[a]		No. of rats	Number (No./cm^2)		Area (mm^2/cm^2)	
				Observed	Net[b]	Observed	Net
1	Trp-P-2	(1/1)	15	26.53±3.51[c]	5.97	2.02±0.41[c]	1.02
	Glu-P-1	(1/1)	18	76.04±8.98[c]	55.48	14.12±8.24[c]	13.12
	MeAαC	(1/1)	16	38.49±13.07[c]	17.93	3.51±1.49[c]	2.51
	AαC	(1/1)	17	29.98±10.58[d]	9.42	1.78±0.66[c]	0.78
	PhIP	(1/1)	15	18.25±2.64	−2.31	0.90±0.36[c]	−0.10
	Trp-P-2	(1/5)	16	25.53±4.25[d]	4.97	1.02±0.37	0.02
	Glu-P-1	(1/5)	15	35.89±12.15[c]	15.33	2.91±1.34[c]	1.91
	MeAαC	(1/5)	15	25.25±7.84	4.69	1.24±0.34[e]	0.24
	AαC	(1/5)	14	23.02±2.75	2.46	1.12±0.54	0.12
	PhIP	(1/5)	16	21.99±4.41	1.43	1.02±0.45	0.02
	Combined	(1/5)	15	62.95±6.86[c,f]	42.39	5.11±1.41[c,f]	4.11
	Trp-P-2	(1/25)	15	23.43±5.86	2.87	0.95±0.29	−0.05
	Glu-P-1	(1/25)	16	26.82±4.15[c]	6.26	1.11±0.33	0.11
	MeAαC	(1/25)	16	23.32±3.97	2.76	0.96±0.30	−0.04
	AαC	(1/25)	16	24.03±2.92[c]	3.47	0.92±0.21	−0.08
	PhIP	(1/25)	15	21.01±4.27	0.45	1.38±1.93	0.38
	Combined	(1/25)	17	28.12±5.77[c]	7.56	1.07±0.33	0.07
2	None		16	20.56±4.00	—	1.00±0.31	—

Data are mean±SD values of foci larger than 0.1 mm in diameter.
a) Relative to the dose levels used in carcinogenicity studies or the re-evaluated dose for Trp-P-2.
b) Values obtained by subtracting the background levels (Group 2).
c) Significantly different from Group 2 at $P<0.001$.
d) Significantly different from Group 2 at $P<0.01$.
e) Significantly different from Group 2 at $P<0.05$.
f) Significantly greater than the sum of 5 individual data at $P<0.05$ (see "Materials and Methods").

parameters. Enhancement in the development of foci by the combined treatment is schematically illustrated in Fig. 2, in which the net values (obtained by subtracting the control value) for the complex mixture are compared with the sums of the individual net values. At the 1/5 dose level, the result for the combined treatment (42.39 foci per cm^2) was greater than the sum of the 5 individual net data (4.97+15.33+4.69+2.46+1.43= 28.88 foci per cm^2). Similarly, the areas of foci (mm^2/cm^2) were increased by the combined treatment (4.11 as against 2.31=0.02+1.91+0.24+0.12+0.02). The respective ratios were 1.47 (42.39/28.89) in number and 1.78 (4.11/2.31) in area. However, the ratios for the 1/25 dose levels were 0.48 (7.56/15.81) and 0.23 (0.07/0.32), respectively, the observed values being much less than those expected from previous data.[23]

In the subgroups without DEN initiation (group 3), very few small foci were found except in the Glu-P-1 case (Table III), where 27.52 foci per cm^2 were induced. MeAαC induced 2.88 foci per cm^2 at the carcinogenic doses and also a few, but large foci in one rat at the 1/5 dose level. Combined treatment with the 5 chemicals at the 1/5 carcinogenic doses induced 7.23 foci per cm^2, and 0.50 foci at the 1/25 dose. A synergistic effect was more clearly observed for the 1/25 combination than for the 1/5 doses in non-initiated rats.

DISCUSSION

Four out of the 5 heterocyclic amines examined in the present study, Trp-P-2, Glu-P-1, MeAαC and AαC, have earlier been demonstrated to be carcinogenic to the liver of rats and/or mice.[12-14, 24-26] PhIP in contrast had been reported not to be carcinogenic to the liver in either mice[28] or rats.[29] The present results obtained with the highest dose levels were thus consistent with the reported liver carcinogenicity for all five chemicals, indicating the reliability of the present system for detection of hepatocarcinogenic potency. Among the chemicals, Glu-P-1 exerted the strongest effect in terms of induction of GST-P-positive foci, the 1/25 dose level still giving significant induction of foci. Numbers or areas of foci in groups given Trp-P-2 or MeAαC at the 1/5 dose level or AαC at the 1/25 dose level were also significantly greater than

Table III. Numbers and Areas of GST-P-positive Foci in the Livers of Rats without Prior DEN Initiation (Group 3)

Treatment (dose)[a]		No. of rats	GST-P-positive foci	
			No./cm^2	mm^2/cm^2
Trp-P-2	(1/1)	10	0.76±0.48	0.01±0.01
Glu-P-1	(1/1)	11	27.52±10.08	1.60±0.69
MeAαC	(1/1)	11	2.88±1.53	0.06±0.04
AαC	(1/1)	10	0.91±0.45	0.01±0.01
PhIP	(1/1)	10	0	0
Trp-P-2	(1/5)	11	0.07±0.15	0.00±0.00
Glu-P-1	(1/5)	11	4.89±3.11	0.14±0.11
MeAαC	(1/5)	11	0.12±0.16	1.04±3.29
AαC	(1/5)	11	0.04±0.13	0.00±0.00
PhIP	(1/5)	11	0.03±0.10	0.00±0.00
Combined	(1/5)	9	7.23±2.48	0.61±0.33
Trp-P-2	(1/25)	11	0	0
Glu-P-1	(1/25)	11	0.06±0.19	0.00±0.00
MeAαC	(1/25)	10	0	0
MαC	(1/25)	11	0	0
PhIP	(1/25)	11	0	0
Combined	(1/25)	11	0.50±0.46	0.01±0.01

Data are mean±SD values for foci larger than 0.1 mm in diameter.

a) Relative to the dose levels used in carcinogenicity studies or the re-evaluated dose for Trp-P-2.

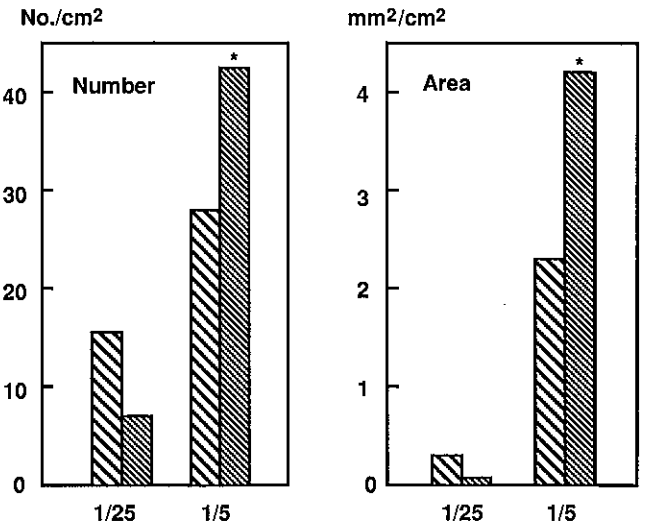

Fig. 2. Effects of combined administration of 5 heterocyclic amines on the induction of GST-P-positive foci in DEN-initiated rats. Synergistic effects regarding both number and area of GST-P-positive foci at the 1/5 dose level are evident, the respective values being significantly greater than the sums of the 5 individual data at $P<0.05$ (*). In this graph, comparison between the combined treatment and individual groups was performed using net values obtained by subtracting the background levels (control values in group 2) from the observed numbers and areas. ▨: simultaneous administration of 5 chemicals. ▧: sum of 5 individual net values.

the control values (group 2). No dose-response effect was seen with PhIP.

Simultaneous treatment of rats with all five heterocyclic amines at 1/5 of the doses used in carcinogenicity studies resulted in apparent synergism in the indution of foci. It is noteworthy that, in a previous experiment using 5 heterocyclic amines other than those used in the present study, 2-amino-dipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-2), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) and 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1), synergistic effects were more evident at the lower dose level (1/25) than the higher one (1/5).[23] This difference in doses between the two experiments may be partly a reflection of different carcinogenic potency of the test chemicals for the liver.

Enhancement or suppression in combined treatment may be caused by various biological activities of chemicals, mainly depending on whether the agents have the same or different sites of primary action (similar or dissimilar) and whether or not the presence of one chemical influences the amounts of the other agents reaching their site of action (interactive or non-interactive). In the case of independent action (dissimilar and non-interactive), the result of combined treatment may be simply additive. Heterocyclic amines, however, are metabolically activated through hydroxylation of the exocyclic amino group mainly by cytochromes P-450 and may induce their own metabolism by inducing increased P-450 levels.[32–34] Although the individual P-450 species involved in metabolic activation and their induction differ between heterocyclic amines,[31] it is likely that the observed synergism in the induction of preneoplastic foci may be partly related to metabolic enzyme induction.[23]

Gart *et al.*[35] introduced two basic possibilities for statistically modeling the response probability in combination groups exposed to two chemicals where the outcome is measured as the proportion of tumor-bearing animals: the additive model and the multiplicative model. They proposed that, under a multistage hypothesis, one would normally expect two carcinogens that act at different stages to act multiplicatively, whereas two carcinogens acting at the same stage might act additively. In the present experiment, possible synergistic effects were evaluated based on the additive model. We defined synergism as the case that the effect of 5 substances acting together exceeds the sum of their effects when acting separately, as reviewed previously.[36]

It is of interest that the sum of the five individual data was usually similar to the average of the five individual data for the five-times-higher dose groups. For instance, the number of foci in the combined-treatment group at the 1/25 dose level was 7.56/cm^2 and the average of the 5 individual data at the 1/5 dose groups was 5.78. The area in the 1/5 combined group was 4.11 mm^2/cm^2 and the average for the 5 individual data for the 1/1 dose groups was 3.47. Although this was not the case with the numbers for the 1/5 combination (42.39 as compared to 17.3) or the area for the 1/25 combination (0.07 as compared to 0.46), a similar correlation was more evident in the previous study.[23] This indicates that a clear dose-response relationship is observed even in the combined treatment, meaning that low dose combinations essentially present much less risk for carcinogenesis than high dose combinations.

Combined effects of five heterocyclic amines were also earlier assessed by Takayama *et al.*[37] in F344 rats using doses set for each chemical at levels 1/5 of those used in previous carcinogenicity studies. Synergism in carcinogenesis was observed not only in the liver but also in the colon, skin, and Zymbal glands. Although precise analysis of synergistic effects was impossible based on the crude proportion data, it is very likely that the combined carcinogenic effects of heterocyclic amines are not limited to the liver. Berger *et al.*[18] also observed combined effects of 3 nitrosamines not only in the liver but also in the gastrointestinal tract, neurogenic tissues, the urinary tract and hematopoietic and lymphatic tissues. However, a well-defined dose-dependency was only apparent for liver tumors. As discussed above, the organotropism of each chemical is important in this context.

Since most human cancers may be caused by trace environmental factors,[3,38] it is of increasing importance that the combined effects of chemicals at relatively low doses be examined. The present data indicate that while individuals are usually exposed to very low doses of environmental chemicals including heterocyclic amines produced in cooking procedures, and while each agent might alone present only low risk, acting in concert they might be capable of inducing tumors. Since a variety of heterocyclic amines may be generated together in any given processed food, the present results, together with those of the previous study, are extremely important. Furthermore, the present experimental system[20,21] may also be of particular use for medium-term analysis of synergistic and summation effects of chemicals. It should be borne in mind that equivalent long-term experiments would be prohibitively expensive given the large number of animals involved.

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REFERENCES

1) Wynder, E. L. and Gori, G. B. Contribution of the environment to cancer incidence: an epidemiologic exercise. *J. Natl. Cancer Inst.*, **58**, 825–832 (1977).
2) Doll, R. Strategy for detection of cancer hazards to man. *Nature*, **265**, 589–596 (1977).
3) Vainio, H., Sorsa, M. and McMichael, A. J. "Complex Mixtures and Cancer Risk," IARC Scientific Publications No. 104 (1990). International Agency for Research on Cancer, Lyon.
4) Miller, A. B. Diet, nutrition and cancer. An epidemiological overview. *J. Nutr. Growth Cancer*, **2**, 159–171 (1985).
5) Doll, R. and Peto, R. The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. *J. Natl. Cancer Inst.*, **66**, 1192–1308 (1981).
6) Scanlan, R. A. Formation and occurrence of nitrosamines in food. *Cancer Res.*, **43**, 2435s–2440s (1983).
7) Miller, E. C. and Miller, J. A. Carcinogens and mutagens that may occur in foods. *Cancer*, **58**, 1795–1803 (1986).
8) Sugimura, T. and Sato, S. Mutagens-carcinogens in foods. *Cancer Res.*, **43**, 2415s–2421s (1983).
9) Sugimura, T. Past, present, and future of mutagens in cooked foods. *Environ. Health Perspect.*, **67**, 5–10 (1986).
10) Sugimura, T. Studies on environmental chemical carcinogens in Japan. *Science*, **233**, 312–318 (1986).
11) Felton, J. S., Knize, M. G., Shen, N. H., Lewis, P. R., Andresen, B. D., Happe, J. and Hatch, F. T. The isolation and identification of a new mutagen from fried ground beef: 2-amino-1-methyl-6-phenylimidazo [4,5-*b*] pyridine (PhIP). *Carcinogenesis*, **7**, 1081–1086 (1986).
12) Matsukura, N., Kawachi, T., Morino, K., Ohgaki, H., Sugimura, T. and Takayama, S. Carcinogenicity in mice of mutagenic compounds from a tryptophan pyrolysate. *Science*, **213**, 346–347 (1981).
13) Sugimura, T. Carcinogenicity of mutagenic heterocyclic amines formed during the cooking process. *Mutat. Res.*, **150**, 33–41 (1985).
14) Ohgaki, H., Hasegawa, H., Kato, T., Suenaga, M., Ubukata, M., Sato, S., Takayama, S. and Sugimura, T. Carcinogenicity in mice and rats of heterocyclic amines in cooked foods. *Environ. Health Perspect.*, **67**, 129–134

(1986).
15) Kuchlbauer, J., Roman, W. and Neumann, H-G. Syn-carcinogenic effects on the initiation of rat liver tumors by *trans*-4-acetylaminostilbene and 2-acetylaminofluorene. *Carcinogenesis,* **6,** 1337–1342 (1985).
16) Hirose, M., Masuda, A., Tsuda, H., Uwagawa, S. and Ito, N. Enhancement of BHA-induced proliferative rat forestomach lesion development by simultaneous treatment with other antioxidants. *Carcinogenesis,* **8,** 1731–1735 (1987).
17) Tatematsu, M., Mutai, M., Inoue, K., Ozaki, K., Furihata, C. and Ito, N. Synergism between sodium chloride and sodium taurocholate and development of pepsinogen-altered pyloric glands: relevance to a medium-term bioassay system for gastric carcinogens and promoters in rats. *Jpn. J. Cancer Res.,* **80,** 1035–1040 (1989).
18) Berger, M. R., Schmähl, 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).
19) Schmahl, W. Synergistic induction of tumours in NMRI mice by combined foetal X-irradiation with low doses and ethylnitrosourea administration to juvenile offspring. *Carcinogenesis,* **9,** 1493–1498 (1988).
20) Ito, N., Tsuda, H., Tatematsu, M., Inoue, T., Tagawa, Y., Aoki, T., Uwagawa, S., Kagawa, M., Ogiso, T., Masui, T., Imaida, K., Fukushima, S. and Asamoto, M. Enhancing effect of various hepatocarcinogens on induction of preneoplastic glutathione S-transferase placental form positive foci in rats — an approach for a new medium-term bioassay system. *Carcinogenesis,* **9,** 387–394 (1988).
21) Ito, N., Imaida, K., Hasegawa, R. and Tsuda, H. Rapid bioassay methods for carcinogens and modifiers of hepatocarcinogenesis. *CRC Crit. Rev. Toxicol.,* **19,** 385–415 (1989).
22) Hasegawa, R., Mutai, M., Imaida, K., Tsuda, H., Yamaguchi, S. and Ito, N. Synergistic effects of low-dose hepatocarcinogens in induction of glutathione *S*-transferase P-positive foci in the rat liver. *Jpn. J. Cancer Res.,* **80,** 945–951 (1989).
23) 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).
24) Hosaka, S., Matsushima, T., Hirono, I. and Sugimura, T. Carcinogenic activity of 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2), a pyrolysis product of tryptophan. *Cancer Lett.,* **13,** 23–28 (1981).
25) Takayama, S., Masuda, M., Mogami, M., Ohgaki, H., Sato, S. and Sugimura, T. Induction of cancers in the intestine, liver and various other organs of rats by feeding mutagens from glutamic acid pyrolysate. *Gann,* **75,** 207–213 (1984).
26) Ohgaki, H., Matsukura, N., Morino, K., Kawachi, T., Sugimura, T. and Takayama, S. Carcinogenicity in mice of mutagenic compounds from glutamic acid and soybean globulin pyrolysates. *Carcinogenesis,* **5,** 815–819 (1984).
27) Takayama, S., Nakatsuru, Y., Ohgaki, H., Sato, S. and Sugimura, T. Atrophy of salivary glands and pancreas of rats fed on diet with amino-methyl-α-carboline. *Proc. Jpn. Acad.,* **61,** Ser. B, 277–280 (1985).
28) Esumi, H., Ohgaki, H., Kohzen, E., Takayama, S. and Sugimura, T. Induction of lymphoma in CDF_1 mice by the food mutagen, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]-pyridine. *Jpn. J. Cancer Res.,* **80,** 1176–1178 (1989).
29) Ito, N., Hasegawa, R., Sano, M., Tamano, S., Esumi, H., Takayama, S. and Sugimura, T. A new colon and mammary carcinogen in cooked food, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). *Carcinogenesis,* **12,** 1503–1506 (1991).
30) Tatematsu, M., Mera, Y., Ito, N., Satoh, K. and Sato, K. Relative merits of immunohistochemical demonstrations of placental A, B, and C forms of glutathione S transferase and histochemical demonstration of γ-glutamyltransferase as markers of altered foci during liver carcinogenesis in rats. *Carcinogenesis,* **6,** 1621–1626 (1985).
31) Rao, C. R. "Linear Statistical Inference and Its Applications," pp. 194–195 (1985). John Wiley & Sons, Inc., New York.
32) Alexander, J. and Wallin, H. Metabolic fate of heterocyclic amines from cooked food. *CRC Crit. Rev. Toxicol.,* **20,** 143–153 (1990).
33) Kato, R. Metabolic activation of mutagenic heterocyclic aromatic amines from protein pyrolysates. *CRC Crit. Rev. Toxicol.,* **16,** 307–348 (1986).
34) Holme, J. A., Wallin, H., Brunborg, G., Sϕderlund, E. J., Hongslo, J. K. and Alexander, J. Genotoxicity of the food mutagen, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP): formation of 2-hydroxyamino-PhIP, a directly acting genotoxic metabolite. *Carcinogenesis,* **10,** 1389–1396 (1989).
35) Gart, J. J., Krewski, D., Lee, P. N., Tarone, R. E. and Wahrendorf, J. "Statistical Methods in Cancer Research. Vol. III. The Design and Analysis of Long-term Animal Experiments," IARC Scientific Publications No. 79, pp. 155–160 (1986). International Agency for Research on Cancer, Lyon.
36) Reif, A. E. Synergism in carcinogenesis. *J. Natl. Cancer Inst.,* **73,** 25–39 (1984).
37) Takayama, S., Nakatsuru, Y. and Sato, S. Carcinogenic effect of the simultaneous administration of five heterocyclic amines to F344 rats. *Jpn. J. Cancer Res.,* **78,** 1068–1072 (1987).
38) Higginson, J. Changing concepts in cancer prevention: limitations and implications for future research in environmental carcinogenesis. *Cancer Res.,* **48,** 1381–1389 (1988).