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Suppressing Effects of 6-(2,5-Dichlorophenyl)-2,4-diamino-1,3,5-triazine and Related Synthetic Compounds on Azoxymethane-induced Aberrant Crypt Foci in Rat Colon

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The modifying effects of dietary administration of 6-(2,5-dichlorophenyl)-2,4-diamino-1,3,5-triazine and 5 related compounds on the occurrence of azoxymethane (AOM)-induced colonic aberrant crypt foci (ACF) were investigated in rats. Male F344 rats were given s.c. injections of AOM (15 mg/kg body weight) once a week for 3 weeks to induce ACF. They also received the diet containing 200 ppm test compound for 5 weeks, starting one week before the first dosing of AOM. At the termination of experiment, all of the compounds had caused a significant reduction in ACF frequency, which might be associated with suppression of the expression of proliferation biomarkers. The apoptotic index in the colonic mucosal epithelium of rats killed at 6 h after the first AOM exposure revealed no blocking activity of the compounds.

Key words: 6-(2,5-Dichlorophenyl)-2,4-diamino-1,3,5-triazine — Triazine derivatives — Aberrant crypt foci — Azoxymethane — Rat

Aberrant crypt foci (ACF) are considered to be preneoplastic lesions, since they are present in carcinogen-treated rodent colon and in the colon of humans with a high risk for cancer development.[1)] This character of ACF favors its use as a biomarker in the identification of cancer modulators.[2)]

Gap junctional intercellular communication is essential for the maintenance of tissue homeostasis and cellular society.[3)] Derangement of this cell-cell interaction causes the growth advantage of initiated cells, and thus intact intercellular communication can work as a tumor-suppressive element. A new anti-ulcer agent, 6-(2,5-dichlorophenyl)-2,4-diamino-1,3,5-triazine (DCPDAT), is reported to increase cyclic-adenosine 5′-monophosphate in the rat gastric mucosa and to upregulate intercellular communication via gap junctions between cultured rabbit gastric endothelial cells.[4,5)] DCPDAT also inhibits *in vivo* angiogenesis in mice.[6)] The effectiveness of combination treatment with DCPDAT and UFT (a mixture of tegafur and uracil in a molar ratio of 1:4) has been reported in metastatic gastric cancer[7)] and inoperable gastric cancer.[8)]

In the present study, possible modifying effects of DCPDAT and its synthetic derivatives (Fig. 1), 6-(2-chlorophenyl)-2,4-diamino-1,3,5-triazine (2CPDAT), 6-(3-chlorophenyl)-2,4-diamino-1,3,5-triazine (3CPDAT), 6-(4-chlorophenyl)-2,4-diamino-1,3,5-triazine (4CPDAT), 6-(4-pyridyl)-2,4-diamino-1,3,5-triazine (PyDAT), and 6-(pyridine *N*-oxide 4-yl)-2,4-diamino-1,3,5-triazine (PyNODAT), on the development of azoxymethane (AOM)-induced colonic ACF were investigated in male F344 rats. The expressions of biomarkers, such as 5-bromo-2′-deoxyuridine (BrdU)-labeling index (BLI) of the colonic epithelial cells, ornithine decarboxylase (ODC) activity of colonic mucosa, and polyamine level in the blood, were also examined. In addition, the apoptotic index (AI) of the colonic epithelium at 6 h after the first AOM injection with or without a test compound was assessed to evaluate the carcinogen-induced damage and the modifying effects of the test chemicals on apoptotic induction.

A total of 147 male F344 rats, 4 weeks old, obtained from Japan SLC Inc., Hamamatsu, were used for this experiment. Animals were housed three or four to a wire cage in an experimental room under controlled conditions of 23±2°C (SD), 50±10% humidity, and 12 h light/dark cycle. They were allowed *ad libitum* access to diet and water. All test chemicals including DCPDAT were synthesized by the Chemotherapy Division, National Cancer Center Research Institute.

After quarantine for 1 week, rats aged 5 weeks were divided into 14 groups as shown in Fig. 2. Rats in groups

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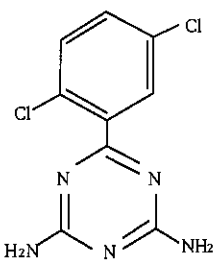
2 through 13 were given the basal diets containing 200 ppm of a test compound for 5 weeks, beginning at 5 weeks of age. Groups 1 and 14 were fed the basal diet CE-2 (Clea Japan, Inc., Tokyo) throughout the study. Starting at 6 weeks of age, animals in groups 1 through 7 were s.c. injected with AOM (15 mg/kg body weight, Sigma Chemical Co., St. Louis, MO) once a week for 3 weeks to induce colonic ACF. Animals were weighed weekly.

At 6 h after the first injection of AOM, 3 rats in all groups were killed to examine the apoptotic change of the colonic mucosa. The removed colons were cut longi-



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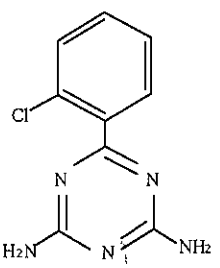

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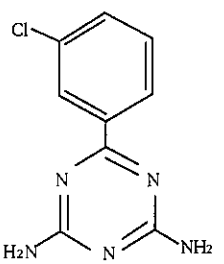

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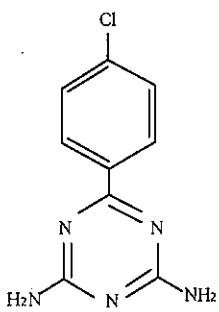

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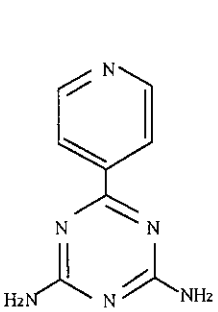
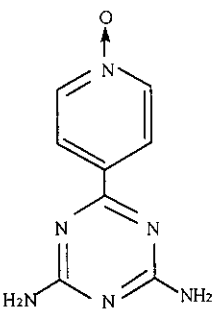


6-(4-pyridyl)-2,4-diamino-1,3,5-triazine



6-(pyridine *N*-oxide 4-yl)-2,4-diamino-1,3,5-triazine

Fig. 1. Molecular structures of the 6 test compounds.

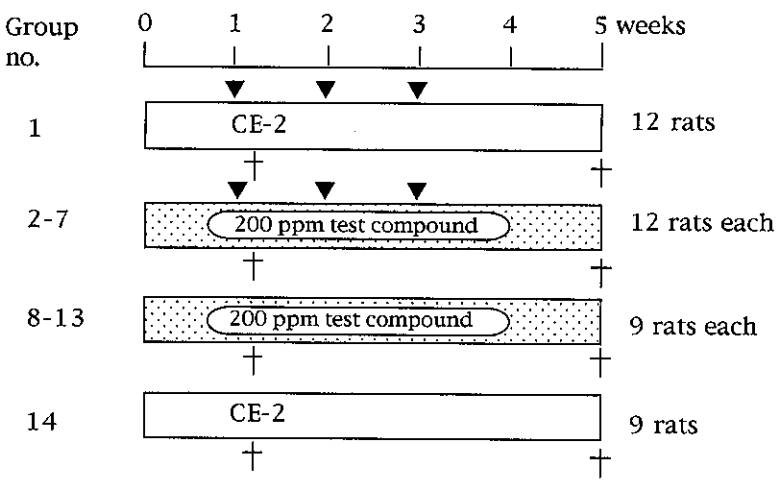


Fig. 2. Experimental protocol. ▼, AOM 15 mg/kg body weight, s.c. injection; ┬, killed. CE-2, basal diet; 0.02% test compound: 6-(2,5-dichlorophenyl)-2,4-diamino-1,3,5-triazine (groups 2 and 8), 6-(2-chlorophenyl)-2,4-diamino-1,3,5-triazine (groups 3 and 9), 6-(3-chlorophenyl)-2,4-diamino-1,3,5-triazine (groups 4 and 10), 6-(4-chlorophenyl)-2,4-diamino-1,3,5-triazine (groups 5 and 11), 6-(4-pyridyl)-2,4-diamino-1,3,5-triazine (groups 6 and 12), and 6-(pyridine *N*-oxide 4-yl)-2,4-diamino-1,3,5-triazine (groups 7 and 13).

tudinally, cleaned, and inflated with 10% neutral buffered formalin. After fixation for 24 h, the whole colon was rolled and embedded in paraffin. Sections (4 μm in thickness) were cut, and stained with hematoxylin and eosin. For the determination of AI, we selected and examined 20 half crypts from the anal side of the colon. The crypts chosen had good shapes with a clearly visible neck, lumen, and base. Apoptotic nuclei induced by AOM were determined according to the criteria proposed by Kerr and Harmon[9] and Majno and Joris.[10] The number and the position of apoptotic cells in each crypt column were determined by counting upwards from the base of the crypt to the mouth. AI was determined by calculating the ratio of apoptotic cells to total number of cells in a crypt.

At the termination of the study, all rats were killed by decapitation. Three rats from each group were randomly selected for the measurement of the ODC activity in the colonic mucosa and polyamine levels in the blood. The remaining animals were used for the detection of colonic ACF and the assessment of BLI. They were given an i.p. injection of BrdU (50 mg/kg body weight, Sigma Chemical Co.) one hour prior to death. The removed colons of the rats used for detecting ACF were cut open longitudinally from cecum to anus, placed between two pieces of filter paper, and fixed in 10% buffered formalin for 24 h. Then, they were stained with 0.5% methylene blue in saline to observe ACF, as described previously.[11] After the observation, they were embedded in paraffin and sliced at 4 μm thickness. The sections were used for histological examination and BrdU immunohistochemistry. For the determination of BLI, BrdU-immunohistochemistry was performed according to the method described previously.[12] Twenty well-shaped crypts of the colonic epithelium were selected and examined, as done in measuring AI. The colons for the measurement of ODC activity were immediately removed, slit open longitudinally, and freed from all contents. The colonic mucosa was scraped off with a knife and stored at -70°C. Proteins were extracted from the mucosa, and ODC activity in the extract was determined by the method described previously.[13] The polyamines in the blood were measured by means of a new enzymatic method developed by Koide *et al.*[14]

The values of body weight, liver weight, relative liver weight (g/100 g body weight), BLI, and the incidences of ACF and apoptosis were compared by use of Student's t test for paired samples or a two-sample t test with Welch's correction.

Mean body and liver weights and relative liver weights in all groups are shown in Table I. The average body weight in group 2 was significantly lower than that in group 1 ($P<0.005$). The mean liver weights in groups 2 and 7 are significantly smaller than that in group 1 ($P<0.03$ and $P<0.05$), and that in group 12 is significantly smaller than that in group 14 ($P<0.05$). In group 7, the relative liver weight was significantly smaller than that in group 1 ($P<0.03$). The relative liver weights of rats in groups 9, 12, and 13 are significantly lower than that in group 14 ($P<0.005$ or $P<0.03$). During this study, no clinical signs of toxicity, low survival, or poor condition were noted. Histologically there was no indication of toxic change in livers or kidneys of the rats given test compounds.

Table I. Body Weights, Liver Weights and Relative Liver Weights of Rats in Each Group

Group no.	Treatment	No. of effective rats	Body weight (g)	Liver weight (g)	Relative liver weight (g/100 g body weight)
1	AOM	9	206±14[a]	11.2±1.6	5.43±0.41
2	AOM+DCPDAT	9	186±11[b]	9.7±0.8[c]	5.22±0.20
3	AOM+2CPDAT	9	207±14	10.2±2.2	4.88±0.82
4	AOM+3CPDAT	9	212±11	11.4±0.7	5.37±0.08
5	AOM+4CPDAT	9	197±14	10.5±0.8	5.30±0.22
6	AOM+PyDAT	9	205±8	11.1±0.7	5.40±0.18
7	AOM+PyNODAT	9	200±7	9.7±1.2[d]	4.83±0.48[e]
8	DCPDAT	5	205±7	10.4±0.7	5.04±0.17
9	2CPDAT	5	207±9	8.9±0.3	4.30±0.06[e]
10	3CPDAT	5	209±10	10.4±0.6	4.99±0.05
11	4CPDAT	5	217±6	10.2±0.7	4.72±0.21
12	PyDAT	5	215±10	9.2±0.8[f]	4.30±0.22[e]
13	PyNODAT	5	212±6	9.2±1.2	4.35±0.46[g]
14	No treatment	5	214±11	10.9±1.3	5.09±0.35

a) Mean±SD.
b–d) Significantly different from group 1 by Student's t test: *b*) $P<0.005$; *c*) $P<0.03$; *d*) $P<0.05$.
e–g) Significantly different from group 14 by Student's t test: *e*) $P<0.005$; *f*) $P<0.05$; *g*) $P<0.03$.

Table II. Effects of Test Compounds on the Development of Aberrant Crypt Foci Induced by AOM

Group no.	Treatment	No. of rats examined	No. of ACF/colon	No. of ACF/cm^2	No. of aberrant crypts/colon	No. of aberrant crypts/focus
1	AOM	6	117±10[a]	12.6±1.5	284±26	2.43±0.17
2	AOM+DCPDAT	6	66±14[b]	6.6±1.8[b]	131±30[b]	2.09±0.17[c]
3	AOM+2CPDAT	6	66±11[b]	5.8±1.5[b]	150±44[b]	2.31±0.34
4	AOM+3CPDAT	6	80±11[b]	6.9±1.1[b]	155±37[b]	1.93±0.26[d]
5	AOM+4CPDAT	6	76±27[c]	6.6±2.9[d]	153±64[b]	1.99±0.20[d]
6	AOM+PyDAT	6	61±8[b]	5.5±0.7[b]	124±18[b]	2.03±0.23[c]
7	AOM+PyNODAT	6	69±19[b]	6.7±2.1[b]	131±35[b]	1.94±0.33[c]
8	DCPDAT	2	0	0	0	0
9	2CPDAT	2	0	0	0	0
10	3CPDAT	2	0	0	0	0
11	4CPDAT	2	0	0	0	0
12	PyDAT	2	0	0	0	0
13	PyNODAT	2	0	0	0	0
14	No treatment	2	0	0	0	0

a) Mean±SD.
b–d) Significantly different from group 1 by Student's *t* test: *b*) $P<0.001$; *c*) $P<0.01$; *d*) $P<0.005$.

Table III. Alteration of Apoptotic Index (AI) and BrdU-Labeling Index (BLI) in the Colonic Epithelium

Group no.	Treatment	AI[a] (No. of rats examined)	BLI[b] (No. of rats examined)
1	AOM	4.38±2.80[c] (3)	11.00±2.89 (4)
2	AOM+DCPDAT	3.76±0.86 (3)	7.98±2.20 (4)
3	AOM+2CPDAT	2.86±0.47 (3)	9.33±1.26 (4)
4	AOM+3CPDAT	3.74±2.69 (3)	9.63±2.03 (4)
5	AOM+4CPDAT	4.86±1.58 (3)	10.12±3.45 (4)
6	AOM+PyDAT	3.00±0.48 (3)	6.59±2.61 (4)
7	AOM+PyNODAT	3.15±1.24 (3)	11.65±3.04 (4)
8	DCPDAT	0.38±0.19 (3)	7.59±2.44 (2)
9	2CPDAT	0.08±0.14 (3)	8.06±0.14 (2)
10	3CPDAT	0.19±0.33 (3)	12.08±3.16 (2)
11	4CPDAT	0.46±0.42 (3)	9.31±0.79 (2)
12	PyDAT	0.07±0.13 (3)	8.30±2.15 (2)
13	PyNODAT	0.20±0.18 (3)	5.50±1.92 (2)
14	No treatment	0.23±0.20 (3)	9.13±3.96 (2)

a) AI (%) was determined at 6 h after the first dosing of AOM.
b) BLI (%) was determined at the termination of the study.
c) Mean±SD.

The ACF data are summarized in Table II. In group 1, AOM-induced ACF at 117±10/rat. The dietary administration of any test compounds caused a significant reduction of the ACF incidence ($P<0.001$ or $P<0.01$). A significant decrease in the number of ACF per cm^2 in groups 2–7 ($P<0.001$ or $P<0.005$) and a significant decrease in the number of aberrant crypts per colon in groups 2–7 ($P<0.001$) were also observed when compared with those in group 1. The treatments with the test compounds significantly reduced the number of aberrant crypts in each focus in all groups except group 3 ($P<0.01$ or $P<0.005$). There were no ACF in the colons of the rats not injected with AOM (groups 8–14).

The incidences of apoptotic cells in the groups at 6 h after AOM treatment and the BLI data are presented in Table III. The incidence of apoptosis in the colonic epithelium in AOM-treated groups was high and the AI in group 1 was 4.38±2.80%. In groups 3, 6, and 7, AIs were slightly less than that in group 1, but there was no significant difference. Without carcinogen treatment, there were few apoptotic nuclei in the colonic crypts. BLIs in groups 2 and 6 were less than that in group 1, but

Table IV. ODC Activity of the Colonic Mucosa and Blood Polyamine Concentrations of Rats in Each Group

Group no.	Treatment	No. of rats examined	ODC activity (pmol $^{14}CO_2$/h/mg protein)		Total polyamine concentration (nmol/mg protein, ±SD)
			Mean±SD	Range	
1	AOM	3	102.8±118.4	31.0–239.5	35.20±1.82
2	AOM+DCPDAT	3	8.8±0.9	8.0–9.7	28.65±0.28[a)]
3	AOM+2CPDAT	3	19.8±6.3	13.9–26.5	29.55±1.58[b)]
4	AOM+3CPDAT	3	28.9±32.6	8.5–66.5	32.09±1.45
5	AOM+4CPDAT	3	20.2±10.8	13.8–32.6	31.21±1.95
6	AOM+PyDAT	3	8.1±4.9	2.9–12.6	29.10±1.27[c)]
7	AOM+PyNODAT	3	4.5±3.4	1.1–7.8	28.03±0.89[d)]
8	DCPDAT	3	8.3±3.2	5.7–11.8	26.29±1.04
9	2CPDAT	3	11.2±9.0	2.9–20.8	25.60±2.61
10	3CPDAT	3	2.2±2.6	0–5.0	26.88±1.19
11	4CPDAT	3	9.2±7.3	3.1–17.3	28.22±3.31
12	PyDAT	3	1.9±1.6	0–3.0	25.23±1.41
13	PyNODAT	3	18.0±12.2	7.1–31.2	27.30±1.61
14	No treatment	2	5.2±0.2	5.1–5.3	29.73±2.54

a) Significantly different from group 1 by Welch's test: $P<0.03$.
b–d) Significantly different from group 1 by Student's *t* test: *b*) $P<0.03$; *c*) $P<0.01$; *d*) $P<0.005$.

there was no significant difference between BLI in group 1 and those in groups 2–7.

The results on ODC activity in the colonic mucosa and polyamine levels in blood are shown in Table IV. Mean ODC activities in groups 2–7 were lower than that in group 1. However, there was no significant difference between the ODC activity in group 1 and those in groups 2–7 because of the large SD values in these groups. Total polyamine levels in groups 2, 3, 6, and 7 were significantly less than those in group 1 ($P<0.03$, $P<0.01$, or $P<0.005$).

The results in the present study indicate that dietary feeding of all test compounds, including DCPDAT, suppressed the occurrence of ACF induced by AOM when administered during the carcinogen exposure. ACF are considered to be possible precursor lesions for colon cancer in rodents and human.[1)] We and other investigators have used this model for screening chemopreventive agents against colon cancer.[11–13)] The results suggest that all compounds tested in this study may have chemopreventive effects on colon carcinogenesis, although a long-term experiment is needed to confirm the present findings.

In the early stage of colon tumorigenesis, the test compounds exhibited no modifying effects on the apoptotic induction by AOM, suggesting that these agents may not have blocking activity.[15)] We recently proposed that different chemopreventive agents may intervene at different stages of carcinogenesis, i.e., the process of formation of carcinogens, the process of activation or detoxification of carcinogens, the process of DNA adduction, the process of oncogene activation, the process of preneoplastic lesion development, and the stage of cancer progression.[16)] It is conceivable that the suppressing effects of the compounds tested in this study may not be associated with carcinogen formation, activation or detoxification, or DNA adduction.

DCPDAT is reported to enhance gap junctional intercellular communication.[5)] Recently, Toriyama-Baba *et al.*[17)] found inhibitory effects of 2CPDAT, 3CPDAT, 4CPDAT and PyNODAT on rat hepatocarcinogenesis using a medium short-term bioassay with the carcinogen diethylnitrosamine (Ito model), but there was no effect in rats fed DCPDAT or PyDAT. Moreover, these compounds did not affect the gap junctional intercellular communication in *in vivo* assay systems. In our recent study, DCPDAT acted as an antipromoter in diethylnitrosamine/phenobarbital-induced hepatocarcinogenesis in rats (manuscript in preparation). Therefore, modifying (inhibitory) effects on carcinogenesis may depend on the target organs and/or carcinogens used, and the suppressing effects may not relate to their ability to modulate intercellular communication, although there are no reports of the effects of 2CPDAT, 3CPDAT, 4CPDAT, PyDAT and PyNODAT on gap junctions in colon and liver. BLI and ODC activity of the colonic epithelium were not modulated significantly by the dietary feeding of the test compounds. However, the administration of the test compounds in groups 2, 3, 6, and 7 reduced polyamine concentration in the blood. Certain chemopreventers could alter the increased polyamine levels induced by carcinogen treatment.[18,19)] Thus, the inhibitory effects of DCPDAT, 2CPDAT, PyDAT, and PyNODAT may be due to the modification of polyamine synthesis.

In summary, dietary administration of DCPDAT and related compounds significantly suppressed the development of AOM-induced rat colonic ACF. Although the mechanisms of these chemopreventive effects are not yet understood, and the toxicity of these compounds needs to be examined, the evidence described here warrants further research on the modifying effects of these compounds using long-term bioassay systems in colon, liver and other organs.

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