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Triazine Derivatives Inhibit Rat Hepatocarcinogenesis but Do Not Enhance Gap Junctional Intercellular Communication

Takaaki Hori,[1] Makoto Asamoto,[2] Vladimir Krutovskikh, Yoshio Iwahori, Mitsuaki Maeda, Hiroyasu Toriyama-Baba, Nobuo Takasuka and Hiroyuki Tsuda

Chemotherapy Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104

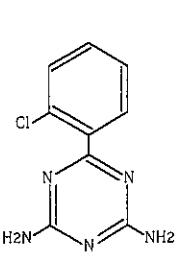
We report here novel candidate chemopreventive agents active against experimental hepatocarcinogenesis. The triazine derivatives 6-(2-chlorophenyl)-2,4-diamino-1,3,5-triazine (2CPDAT), 6-(3-chlorophenyl)-2,4-diamino-1,3,5-triazine (3CPDAT), 6-(4-chlorophenyl)-2,4-diamino-1,3,5-triazine (4CPDAT), 6-(4-pyridyl)-2,4-diamino-1,3,5-triazine (PyDAT), and 6-(pyridine *N*-oxid-4-yl)-2,4-diamino-1,3,5-triazine (PyNODAT), synthesized in our laboratory, in addition to 6-(2,5-dichlorophenyl)-2,4-diamino-1,3,5-triazine (DCPDAT), or irsogladine, which is a widely used anti-ulcer drug, were investigated for potential chemopreventive effects in a rat liver medium-term bioassay system. A significant inhibitory influence on enzyme-altered liver foci was found for 2CPDAT, 3CPDAT, 4CPDAT, and PyNODAT, but not for DCPDAT or PyDAT. The involvement of gap junctional intercellular communication in the inhibition was studied, but no change in gap junctional intercellular communication capacity in rat liver cells *in vitro* or in gap junction protein (connexin 32) expression in rat liver *in vivo* was noted. These results indicate that, although these irsogladine analogues exert inhibitory effects on rat liver carcinogenesis, their action is independent of modification of gap junctional intercellular communication.

Key words: Triazine — Chemoprevention — Rat — Liver

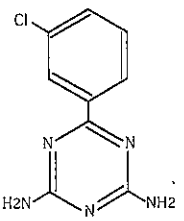
6-(2,5-Dichlorophenyl)-2,4-diamino-1,3,5-triazine (DCPDAT), or irsogladine, is clinically used as an anti-ulcer drug which may act by increasing gastric mucosal blood flow without inhibition of secretion of gastric juice, acid or pepsin.[1] This triazine derivative inhibits the induction of plasminogen activator synthesis in endothelial cells and also inhibits angiogenesis.[2] It is also known to have the ability to enhance gap junctional intercellular communication in epithelial cells of rabbit stomach mucosa.[3] Recently, involvement of gap junctional intercellular communication (GJIC) in carcinogenesis has become well established.[4,5] For example, GJIC is inhibited by certain tumor-promoting agents such as 12-O-tetradecanoylphorbol-13-acetate (TPA),[6,7] 1,1-*bis*(*p*-chlorophenyl)-2,2,2-trichloroethane (DDT),[8–10] benzoyl peroxide (BP),[11] L-ethionine and clofibrate.[12] The sensitivity of gap junction assay was reviewed recently for about 300 substances with various biological activities and it was found that these assays showed 60% positivity for detection of tumor promoters or carcinogens.[13] Conversely, some chemopreventive agents, e.g., α, β-carotene, lutein, canthaxanthin, and lycopene, have been shown to enhance intercellular communication via gap junctions.[14] With regard to chemopreventive effects in the stomach, few studies have been performed, and the role, if any, that GJIC plays in stomach carcinogenesis has yet to be elucidated. Far more is known, however, from the viewpoint of medium-term bioassay systems,[15,16] and the alteration of GJIC during carcinogenesis in the rat liver is also well established.[17] Therefore, we studied the influence of irsogladine (Fig. 1) on hepatocarcinogenesis using an established bioassay system.[15,16] We also investigated the effects of the 3 triazine derivatives (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)) to address the question of which chlorine atom of the benzene ring of 6-(2,5-dichlorophenyl)-2,4-diamino-1,3,5-triazine (DCPDAT) is responsible for the activity. 6-(4-Pyridyl)-2,4-diamino-1,3,5-triazine (PyDAT) and 6-(pyridine *N*-oxid-4-yl)-2,4-diamino-1,3,5-triazine (PyNODAT), which has a hydrophilic site and reduced basicity at the nitrogen atom due to *N*-oxidation, were also investigated to check the stereochemical requirements for activity and the effect of a basic site in the molecule. To determine whether or not these derivatives affect gap junctional intercellular communication, as observed with other chemopreventive agents, an assessment of their influence on GJIC capacity in a rat liver epithelial cell line (IAR 20),[18] and on the expression of the major liver gap junctional protein, connexin 32, in rat liver *in vivo*[19] was included.

[1] Present address; First Department of Pathology, Osaka City University Medical School, 1-4-54 Asahi-cho, Abeno-ku, Osaka 545

[2] To whom all correspondence should be addressed.



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



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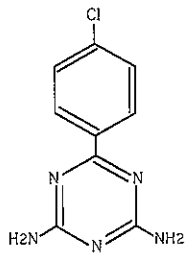
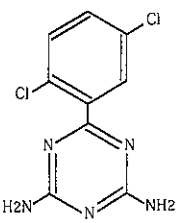
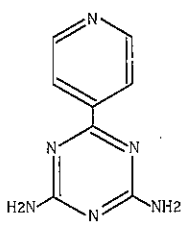


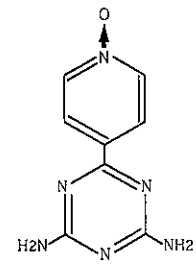
6-(4-chlorophenyl)-2,4-diamino-1,3,5-triazine (4CPDAT)



6-(2,5-dichlorophenyl)-2,4-diamino-1,3,5-triazine (DCPDAT) (irsogladine)



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



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

Fig. 1. Structures of the diamino-*s*-triazine derivative test chemicals.

MATERIALS AND METHODS

Animals and experimental schedule Male 7-week-old F344/DuCrj rats (Charles River Japan Inc., Atsugi) were used. They were housed in plastic cages in an air-conditioned room at 24±2°C, 60±5% humidity. A total of 175 rats were intraperitoneally injected with 20 mg/kg body weight of diethylnitrosamine (DEN) and, starting 2 weeks later, then fed diets (Oriental MF, Oriental Yeast Co., Ltd., Tokyo) containing test compounds *ad libitum* for 6 weeks: 0.0182% 2CPDAT, 0.0182% 3CPDAT, 0.0182% 4CPDAT, 0.02% DCPDAT, 0.0164% PyDAT, 0.0172% PyNODAT, or no supplement as a control. The dose of DCPDAT was determined in accordance with published date for the subacute toxicity,[20] which indicated that a 0.02% dose was not toxic. The concentrations of other derivatives were adjusted to be equimolar to 0.02% DCPDAT. All the test compounds were synthesized in our laboratory,[21-24] and their purity was over 99.95% by elemental analysis. They are stable in the diet. Partial hepatectomy was performed at the end of week 3 and all rats were killed for quantitation of glutathione *S*-transferase placental form (GST-P)-positive preneoplastic lesions in the liver at the end of week 8.[25]

For the study of connexin 32 expression, male 7-week-old F344/DuCrj rats (Charles River Japan) were treated with the 6 triazine derivatives at the same dietary dose levels as for the medium-term bioassay, for one week. The livers were then excised, cut into 4–5 mm thick slices, frozen in isopentane prechilled to −130°C in a liquid nitrogen bath, and stored at −80°C until use.

Immunohistochemistry for GST-P Liver tissue slices were fixed in acetone and embedded in paraffin. Sections cut at 3–4 μm were deparaffinized in xylene, and hydrated through a graded series of alcohols before treatment with rabbit anti-rat polyclonal antibody against GST-P[25] at a dilution of 1 : 5000. Binding sites were demonstrated by the avidin-biotin-peroxidase complex (ABC) method (Vector Laboratories, Inc., Burlingame, CA) using diaminobenzidine-H_2O_2 (the sections were lightly counterstained with hematoxylin). The numbers and areas of GST-P-positive lesions >0.1 mm diameter per cm^2 liver specimen were assessed using an Image Analyzer (Olympus-Ikegami VIP-21C, Olympus Co., Ltd., Tokyo).

Cell culture and gap junctional communication assay Cells of the rat liver epithelial cell line IAR 20 were obtained from the Japanese Cancer Research Resources Bank and routinely subcultured in tissue culture flasks in

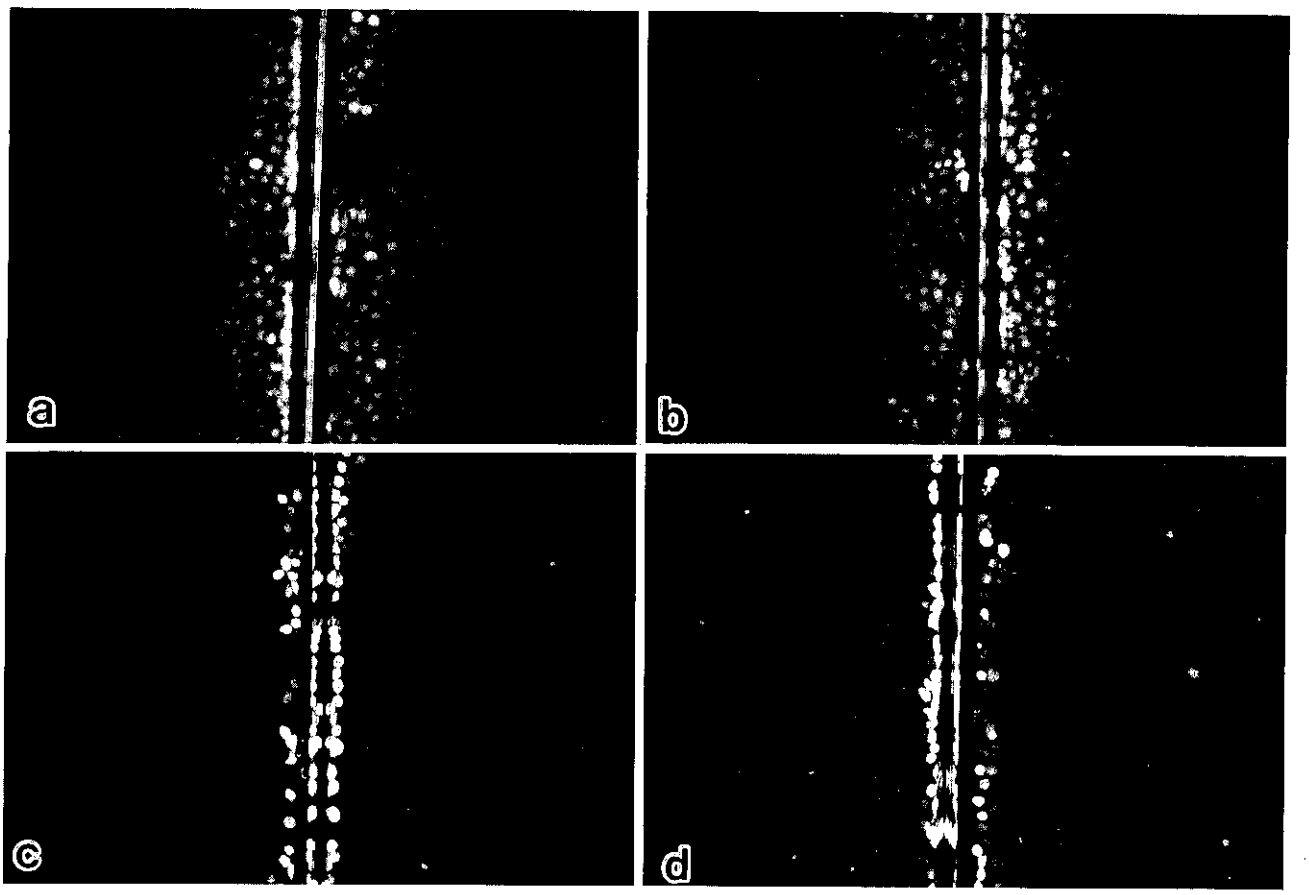


Fig. 2. Gap junctional intercellular communication capacity of IAR 20 cells revealed by the scrape dye loading method. a, treated with solvent (DMSO) only; b, treated with PyNODAT; c, treated with DMSO and TPA; d, treated with PyNODAT and TPA. Dramatic inhibition of gap junctional intercellular communication is evident with TPA, whereas PyNODAT appears to have no effect, with or without TPA treatment.

Williams' medium E, supplemented with 10% heated-inactivated fetal bovine serum, 5.84 mg/ml L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (GIBCO BRL, Life Technologies, Inc., Grand Island, NY). Subconfluent cells were treated for three days with one of the 6 test chemicals dissolved in dimethyl sulfoxide (DMSO) at 100 n*M* concentration. After a 1 h exposure to 25 ng/ml TPA (Sigma, St. Louis, MO), or without such exposure, the GJIC activity was measured as follows. Cells were rinsed in PBS, then lines of cells were scraped off using a sharp blade in PBS containing 20 mg/ml Lucifer Yellow (Sigma). The preparations were incubated in the dark for 10 min at room temperature, the Lucifer Yellow was removed and the cultures were washed several times in PBS. The cultures were then photographed under fluorescence illumination.[26] In order to evaluate GJIC quantitatively, the number of rows labeled with the fluorescent dye was counted at ten sites as illustrated in Fig 2.[27] Statistical analysis was performed using Student's *t* test.

Immunohistochemical staining for Cx 32 Frozen sections (5 μm) were fixed in acetone cooled to -20°C for 5 min, then incubated for 1 h in 1% skim milk in PBS, and treated with rabbit polyclonal antibody against Cx 32[17] at a dilution of 1 : 6000. The binding sites were visualized with biotinylated anti-rabbit IgG and FITC-conjugated streptavidin (Vector Laboratories, Inc.).

RESULTS

Numbers and areas of GST-P-positive liver lesions Animals treated with the triazine derivatives showed no sign of toxicity, such as body weight loss.

Table I summarizes data on the numbers and areas of GST-P-positive lesions in triazine derivative-treated and control rats. 2CPDAT, 3CPDAT, 4CPDAT and

Table I. Quantitative Data for GST-P-positive Liver Lesions

Treatment	No. of rats	No. of foci per cm^2	Area of foci (mm^2/cm^2 ($\times 10^{-3}$))
2CPDAT	23	0.54 ± 0.61[a] (62)[b]	6.91 ± 9.79[a] (78)[b]
3CPDAT	24	0.63 ± 0.55[a] (56)	7.79 ± 7.55[a] (75)
4CPDAT	23	0.70 ± 0.67[a] (51)	9.60 ± 9.08[a] (69)
DCPDAT	23	1.37 ± 0.89 (4)	16.21 ± 10.96 (47)
PyDAT	23	1.35 ± 0.9 (5)	17.27 ± 12.83 (44)
PyNODAT	25	0.48 ± 0.40[a] (66)	5.81 ± 4.72[a] (81)
Control	15	1.42 ± 1.39	30.80 ± 42.19

a) This value is significantly different from that for control rats (Student's *t* test, $P<0.05$).

b) Percent specific inhibition: $\frac{\text{control value}-\text{experimental value}}{\text{control value}}\times 100$.

2CPDAT, 6-(2-chlorophenyl)-2,4-diamino-1,3,5-triazine.
3CPDAT, 6-(3-chlorophenyl)-2,4-diamino-1,3,5-triazine.
4CPDAT, 6-(4-chlorophenyl)-2,4-diamino-1,3,5-triazine.
DCPDAT (irsogladine), 6-(2,5-dichlorophenyl)-2,4-diamino-1,3,5-triazine.
PyDAT, 6-(4-pyridyl)-2,4-diamino-1,3,5-triazine.
PyNODAT, 6-(pyridine *N*-oxid-4-yl)-2,4-diamino-1,3,5-triazine.

PyNODAT exerted significant inhibitory effects as evidenced by decreased numbers and areas of foci ($P<0.05$). DCPDAT (irsogladine) and PyDAT did not show statistically significant inhibition.

Quantitative analysis of GJIC by scrape-loading/dye transfer To measure GJIC capacity, the scrape-loading dye transfer technique was used. Lucifer Yellow does not diffuse through intact plasma membranes and its low molecular weight permits its transmission from one cell to another across gap junctions. The dye is loaded into cells which are damaged by scraping with a blade, and the dye spreads through gap junctions.

In untreated IAR 20 cells, the dye spread quite well from the scraped line with the average number of rows of stained cells being 7.0 ± 1.2. None of the six triazine derivatives altered the communication capacity. In contrast, when the cells were exposed to TPA, communication was dramatically inhibited to an average of 1.2 ± 0.4 rows of stained cells. The triazine derivatives did not affect this TPA inhibition (Fig. 2).

Expression of connexin 32 protein Connexin 32 was intensely stained on the cell membranes of rat liver tissue. However, no significant difference in the number or size of immunohistochemically demonstrated Cx 32 spots was evident between the treated and untreated liver cases.

DISCUSSION

The present study showed that the mechanism by which triazine derivatives inhibit hepatocarcinogenesis does not involve alteration of the capacity for cell-cell communication. In this study, no toxic effects by the triazine derivatives were noted, so the inhibition of hepatocarcinogenesis by the triazine derivatives was not due to toxicity. Irsogladine increases gap junctional intercellular communication through an increase of cyclic AMP in cultured rabbit gastric epithelial cells,[3] and cyclic AMP itself delays the disappearance of gap junctions between pairs of rat hepatocytes in primary culture.[28] GJIC may physiologically control the cell cycle and growth by mediating transfer of signal-transducing substances which are smaller than 1000 Da.[29] Certain tumor-promoting agents can inhibit gap junctional intercellular communication and this inhibition may be involved in the clonal expansion of initiated cells by releasing them from the suppressive control exerted by surrounding normal cells.[6, 30] Many cancer cells have decreased numbers of gap junctions and loss of intercellular communication has been correlated with the degree of malignancy of tumors.[4, 5, 31, 32] Conversely, several putative chemopreventive agents can up-regulate gap junctional intercellular communication capacity.[33–35]

Clinically, irsogladine is widely used as an anti-ulcer agent, with few side effects. There is also a report of complete disappearance of metastatic abdominal tumors from gastric cancer after treatment with this drug.[36] This is the rationale for the present testing of triazine derivatives, including irsogladine, for chemopreventive capacity using an established rat liver medium-term bioassay system. Remarkable inhibition of the development of GST-P-positive preneoplastic lesions was observed with 2CPDAT, 3CPDAT, 4CPDAT and PyNODAT and weak (not statistically significant) inhibition was seen with DCPDAT and PyDAT. However, no relationship between the structures of these derivatives and the activity was apparent. Recently, suppressive effects of these triazine compounds on the development of azoxymethane-induced aberrant crypt foci in rat colon were re-

ported.[37] Although they appear to be good candidates for novel chemopreventive agents, the mechanisms underlying these inhibitory effects are still unclear and from the present results would appear to involve modulation of processes other than gap junctional communication.

In the normal rat liver, connexin 32 protein is a major gap junction protein,[19] but the IAR 20 cells which we used in our *in vitro* gap junction assay express the related connexin 43, despite being derived from liver.[38] While connexin 43 may not be a target of these triazine derivatives, the lack of any effect on immunohistochemically detected levels implies that GJIC did not play a role in the activity of these agents. However, if a chemical affects the gating of gap junction channels, this may not be detected by immunohistochemistry. Recently an *in vivo* gap junction assay for rat liver was developed,[17] and application of this technique to the present model should allow us to obtain a definitive answer to the question of whether gap junction channels play any role in the inhibition of hepatocarcinogenesis by triazine derivatives. Another possible mechanism of cancer prevention is inhibition of angiogenesis, and in this connection, it is noteworthy that irsogladine exhibits such activity.[2]

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