Jpn. J. Cancer Res. **89**, 371–376, April 1998

Suppressive Effect of Irsogladine Maleate on Diethylnitrosamine-initiated and Phenobarbital-promoted Hepatocarcinogenesis in Male F344 Rats

Shigeyuki Sugie,[1, 2, 5] Kiyohisa Okamoto,[1] Fusao Ueda,[3] Tomoyuki Watanabe,[1] Takuji Tanaka[4] and Hideki Mori[1]

[1]Institute of Laboratory Animals, [2]Department of Pathology, Gifu University School of Medicine, 40 Tsukasa-machi, Gifu 500-8705, [3]Research Laboratories, Nippon Shinyaku Co., Ltd., Nishiohji-Hachijo, Minami-ku, Kyoto 601 and [4]Department of Pathology, Kanazawa Medical University, Uchinada-cho, Ishikawa 920-0263

Modifying effects of irsogladine maleate (IRG) on diethylnitrosamine (DEN)-induced hepatocarcinogenesis were examined in male F344 rats. Six-week-old rats were divided into 8 groups. Groups 1 through 4 were given a single i.p. injection of DEN (200 mg/kg body weight) at the start of the experiment, whereas groups 5 through 8 received a single i.p. injection of saline as the vehicle treatment. Groups 1 and 8 were kept on the basal diet and distilled water throughout the experiment (36 weeks). Groups 2 and 7 were exposed to 500 ppm phenobarbital (PB) in the drinking water, starting one week after the carcinogen or vehicle treatment. Groups 3 and 5 were fed the diet mixed with 125 ppm IRG from one week after DEN or vehicle treatment. Groups 4 and 6 were given 125 ppm IRG-containing diet and drinking water with 500 ppm PB after the carcinogen or vehicle treatment. Liver neoplasms developed in groups 1 (1/15 rats, 7%) and 2 (14/14 rats, 100%). However, no liver tumors were found in rats of groups 3 through 8. Incidence and average number of liver neoplasms in group 4 (0/14 rats, 0%) were less than those in group 2 ($P<0.0001$). The number of glutathione *S*-transferase placental form (GST-P)-positive liver cell foci in group 3 or 4 was significantly smaller than that in the appropriate control ($P<0.01$, $P<0.001$, respectively). The average and unit areas of these foci in group 4 were also significantly smaller than those in group 2 ($P<0.001$, $P<0.05$, respectively). These results suggest that IRG could be a chemopreventive agent for rat liver carcinogenesis.

Key words: Irsogladine maleate — Hepatocarcinogenesis — Chemoprevention

Chemoprevention embraces the concept that non-carcinogenic synthetic chemicals or natural products can inhibit the process of carcinogenesis. A number of agents have proved effective against chemical carcinogenesis in different organs,[1, 2] and have been classified into two major categories, i.e., blocking and suppressing agents.[1]

The mechanism of carcinogenesis is considered to involve multistep genetic alterations. Using animal models, the stages of initiation, promotion and progression have been well investigated, and in particular, studies on hepatocarcinogenesis have provided considerable information.[3–6] Gap junctional intercellular communication (GJIC) has been reported to play an important role in the control of cell proliferation or differentiation,[7–9] and the modulation of GJIC is suggested to be involved in hepatocarcinogenesis, especially in the promotion phase.[8, 10] The gap junctions mediate the transfer of signal-transducing factors such as calcium, cAMP, and inositol triphosphate.[11] They are made up of oligomeric proteins composed of 6 subunits, called connexins (Cxs), and more than 10 connexins, are so far known in mammals. Among them, Cx 32 and Cx 26 are commonly expressed in hepatocytes.[12, 13] Furthermore, Cx 32 is known to decrease in preneoplastic and neoplastic lesions of the liver, while Cx 26 is reported to show a different expression pattern from that of Cx 32 in preneoplastic and neoplastic lesions.[14] Previously, we have demonstrated that *in vivo* exposure to liver tumor promoters, such as phenobarbital (PB) or dichlorodiphenyltrichloroethane, decreased the size and altered the distribution of gap junctions in rat hepatocytes, suggesting inhibitory effects of these agents on GJIC.[15] Agents that promote the function of gap junctions may have suppressing effects on carcinogenesis.[8]

Recently, irsogladine maleate (IRG) (Fig. 1), an antiulcer agent, has been found to enhance GJIC,[16–18] and combination chemotherapy with IRG and other agents was reported to be effective against metastatic tumors from gastric cancer.[19] We have immunohistochemically examined the effect of IRG on gap junctions of the liver, and found that IRG inhibited the decrease of Cx 32 induced by PB (unpublished data).

In the present study, we examined the possible modifying effect of IRG in a rat hepatocarcinogenesis model using diethylnitrosamine (DEN) as an initiator. Dose

[5] To whom requests for reprints should be addressed at the Department of Pathology, Gifu University School of Medicine.

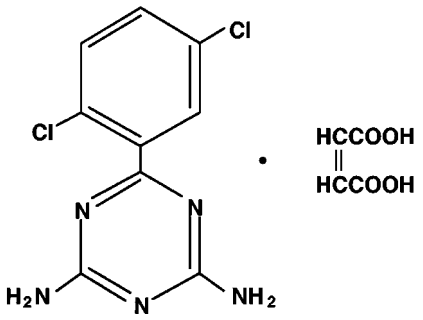

Fig. 1. Molecular structure of irsogladine maleate ($C_{12}H_{11}C_{12}N_5$-O_4: 372.17).

selection of IRG was based on maximum tolerated dose (MTD) values in the previous study.[20] IRG was administered during the promotion phase, and glutathione *S*-transferase placental form (GST-P)-positive preneoplastic lesions were analyzed.

MATERIALS AND METHODS

Animals, diet, drinking water and carcinogen Weanling male F344 rats, purchased from Shizuoka SLC Co., Shizuoka, were used. Powdered CE-2 (Clea Japan Inc., Tokyo) was used as a basal diet. DEN and PB were purchased from Nacalai Tesque Inc., Kyoto and Maruishi Pharm. Co., Osaka, respectively. IRG was synthesized by Nippon Shinyaku Co., Inc., Kyoto.

All animals were housed in wire cages (3 rats/cage). They had free access to water and diet under controlled environmental conditions of humidity (50±10%), lighting (12 h light/dark cycle) and temperature (23±2°C). The experimental diet mixed with IRG and drinking water containing PB were prepared weekly and stored in a cold room (4°C).

Experimental procedure A total of 132 rats, 6 weeks of age, were divided into 8 groups (Fig. 2). Animals of groups 1 through 4 received a single i.p. injection of DEN (200 mg/kg body weight) at the start of the experiment. Groups 5 through 8 were given a single i.p. injection of saline (vehicle). Rats of groups 1 and 8 were fed the basal diet alone throughout the experiment (36 weeks). Groups 2 and 7 were given drinking water containing 500 ppm PB from one week after the carcinogen or vehicle treatment. Groups 3 and 5 were fed the diet mixed with 125 ppm IRG from one week after DEN or vehicle treatment. Groups 4 and 6 were given 125 ppm IRG-containing diet and drinking water with 500 ppm PB from one week after the carcinogen or vehicle treatment. At 11 and 21 weeks after the carcinogen treatment, 3 rats from each group were killed to determine the incidence of altered liver cell foci positive for GST-P. At the termination of the experiment (36 weeks), remaining animals were killed by exposure to ether. At autopsy, the location, number and size of liver tumors were recorded. Liver tissues were sliced into 2 pieces from each sublobe. One set of slices was fixed in cold acetone and another set was fixed in 10% buffered formalin, embedded in paraffin blocks, and processed for routine histological observation with the use of hematoxylin and eosin stain. The other organs were also subjected to routine histological processing for histopathological examination.

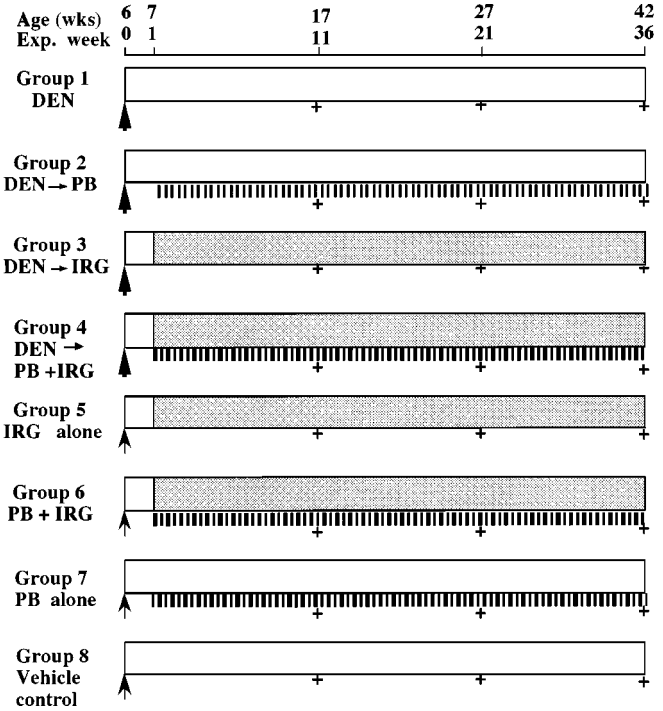

Fig. 2. Experimental design. ▲: DEN 200mg/kg body weight i.p. injection. ▲: Saline. ▮▮▮▮: PB 500 ppm in drinking water. □: Basal diet. +: Animals killed. ▨: 125 ppm IRG.

Immunohistochemical staining The liver sections from acetone-fixed tissues were stained for GST-P using the avidin-biotin-peroxidase complex method (Vectastain ABC kit, Vector Lab. Inc., Burlingame, CA).[21] Anti-GST-P antibody was provided by Dr. K. Satoh.[22, 23] The areas of GST-P-positive foci and the number of foci/cm^2 were measured by means of an image analyzer (IPAP) with a microscope (Sumitomo Chemical Co., Ltd., Osaka). GST-P-positive lesions composed of more than 11 cells were considered as altered liver cell foci.

Statistical analysis Differences of incidence or density of pathological lesions in the liver between groups were

Table I. Body and Relative Liver Weights of F344 Rats Treated with DEN, PB and/or IRG

Treatment	No. of rats (initial)	Body and relative liver weights at:					
		11 wks		21 wks		36 wks	
		Body weight (g)	Relative liver weight(%)	Body weight (g)	Relative liver weight(%)	Body weight (g)	Relative liver weight(%)
DEN alone	21	292±2[a)]	3.43±0.03	330±11	3.33±0.20	378±44	3.42±0.20
DEN+PB	21	317±5[b)]	4.53±0.13[c)]	337±3	4.25±0.12[b)]	342±36[d)]	4.80±0.25[c)]
DEN+IRG	21	274±7[d)]	3.53±0.10	310±8	3.22±0.18	281±20[c)]	3.14±0.28[b)]
DEN+PB+IRG	21	289±3[e)]	3.92±0.61	281±31[f)]	4.08±0.33	288±40[g)]	4.25±0.23[h)]
IRG alone	12	292±41	3.23±0.37	315±15	3.27±0.49	333±15	3.04±0.15
PB+IRG	12	286±24	4.91±0.13	298±11	4.82±0.34	303±12	4.28±0.18
PB alone	12	311±9	4.72±0.08	327±19	4.68±0.11	342±24	4.57±0.34
Vehicle control	12	278±10	3.11±0.13	325±18	3.15±0.15	397±27	3.22±0.24

a) Mean±SD.
b–d) Significantly different from the rats treated with DEN alone by Student's *t* test (*b*) $P<0.005$, *c*) $P<0.0001$, *d*) $P<0.05$).
e–h) Significantly different from the rats treated with DEN+PB by Student's *t* test (*e*) $P<0.005$, *f*) $P<0.05$, *g*) $P<0.001$, *h*) $P<0.0001$).

analyzed by the χ^2-test, Fisher's exact probability test or Student's *t* test.

RESULTS

General observation There was no clear evidence of toxicity in the animals exposed to the IRG diet (Table I). Two rats in group 1 and one rat in group 2 died of pneumonia before the termination of the experiment, and no neoplasms were found in them. Mean body weights of the animals given the basal diet alone (group 1), IRG diet alone (group 5), PB alone (group 7), and PB and IRG (group 6) were respectively 397±27g, 333±15g, 342±24g and 303±12g at the termination. Mean relative liver weights of the groups treated with PB were larger than those of the groups without PB treatment, though the differences were not statistically significant. In other organs, no apparent toxic effects were found in IRG-treated groups.

Tumor incidence Liver tumors were recognized only in groups 1 and 2. They were all of hepatocellular origin (Table II). Hepatocellular adenomas were found in both groups, but liver cell carcinomas were seen only in group 2. Interestingly, no hepatocellular neoplasms were present in any group exposed to IRG. The incidence of hepatocellular carcinoma or adenoma, and the incidence of total liver neoplasms of group 2 were significantly higher than those of the other groups ($P<0.01$).

Incidence of hepatocellular foci In this study, three types of preneoplastic hepatocellular foci (clear, eosinophilic and basophilic), which were also positive for GST-P, were found in the groups exposed to DEN. The results of quantitative analysis on the frequency of GST-P-positive foci are summarized in Table III. GST-P-positive foci were seen in all the DEN-treated animals. The density and average area of GST-P-positive foci of group 2 were higher than those of group 1 or group 4 (Table III). The density of GST-P-positive foci of group 3 was rather lower than that of group 1, although the difference was not significant. A significant decrease in the average area of GST-P-positive foci was present in the animals fed the IRG diet compared to the appropriate positive controls ($P<0.001$ or $P<0.0001$). The area of GST-P-positive foci was significantly decreased in the groups given IRG ($P<0.0001$).

DISCUSSION

The results of the present study clearly indicate the inhibitory effect of IRG on DEN-induced rat hepatocarcinogenesis in rats when given during the promotion phase. In this study, IRG showed a prominent suppressive effect on hepatocarcinogenesis promoted by PB, suggesting that IRG basically acted as an antipromoter. Quantitative analysis of the altered liver cell foci using the phenotypic marker GST-P was consistent with the incidences of liver neoplasms. These liver cell foci are generally recognized as preneoplastic lesions in the lineage of hepatocellular carcinoma development and are considered to reflect the carcinogenic potential, due to the consistent manner in which liver cell foci appear during the post-initiation stage of hepatocarcinogenesis.[24)]

IRG has been reported to enhance GJIC.[16–18)] An inverse correlation between the expression of Cx 32 and BrdU labeling index has been reported in partially hepatectomized liver.[8, 9)] Reduction of GJIC is suggested to be associated with the process of mitosis.[25)] Thus, modulation of carcinogenesis by GJIC is considered to be important, especially in the promotion phase.[8)] Tsuda *et al*. reported a progressive decrease of Cx 32 expression in liver carcino-

Table II. Incidence of Hepatocellular Neoplasms in Each Group

Treatment	No.of rats (terminal)	Incidence(%)			Multiplicity		
		Hepato-cellular neoplasm	Adenoma	Carcinoma	Hepato-cellular neoplasm	Adenoma	Carcinoma
DEN alone	13	1(7)	1(7)	0	0.07±0.25[a)]	0.07±0.25	0
DEN+PB	14	14(100)[b)]	9(64)[c)]	7(50)[d)]	2.64±2.01[e)]	1.73±1.71[f)]	0.82±1.11[g)]
DEN+IRG	15	0	0	0	0	0	0
DEN+PB+IRG	15	0[h)]	0[i)]	0[j)]	0[k)]	0[l)]	0[m)]
IRG alone	6	0	0	0	0	0	0
PB+IRG	6	0	0	0	0	0	0
PB alone	6	0	0	0	0	0	0
Vehicle control	6	0	0	0	0	0	0

a) Mean±SD.
b–d) Significantly different from the rats treated with DEN alone by Fisher's exact probability test (*b*) $P<0.0001$, *c*) $P<0.01$, *d*) $P<0.005$).
e–g) Significantly different from the rats treated with DEN alone by Student's *t* test (*e*) $P<0.0005$, *f*) $P<0.005$, *g*) $P<0.05$).
h–j) Significantly different from the rats treated with DEN alone by Fisher's exact probability test (*h*) $P<0.0001$, *i*) $P<0.005$, *j*) $P<0.005$).
k–m) Significantly different from the rats treated with DEN + PB by Student's *t* test (*k*) $P<0.0005$, *l*) $P<0.005$, *m*) $P<0.05$).

Table III. Results of the Quantitative Analysis of GST-P-positive Foci in Rats of Each Group

Treatment	GST-P positive foci					
	11 wks		21 wks		36 wks	
	Density (/cm^2)	Average area ($\times 10^4\ \mu$m^2)	Density (/cm^2)	Average area ($\times 10^4\ \mu$m^2)	Density (/cm^2)	Average area ($\times 10^4\ \mu$m^2)
DEN alone	0.89±0.70[a)]	2.28±1.33	1.13±0.75	3.21±2.34	3.79±0.63	10.95±7.76
DEN+PB	7.15±2.75	2.68±4.68	14.22±5.49	5.29±6.24	19.30±1.68[b)]	21.87±9.77[b)]
DEN+IRG	0.55±0.26	1.89±1.57	0.92±0.40	3.71±4.05	3.40±1.34	7.80±7.71[c)]
DEN+PB+IRG	2.79±1.61	2.52±2.95	5.20±1.36	2.43±2.64	8.15±3.89[d,e)]	8.92±6.32[e)]
IRG alone	0	0	0	0	0	0
PB+IRG	0	0	0	0	0.27±0.30	6.72±1.57
PB alone	0	0	0.12±0.08	—	0.60±0.59	8.45±4.10
Vehicle control	0	0	0	0	0.23±0.41	4.02±0.26

a) Mean±SD.
b,c) Significantly different from the rats treated with DEN alone by Student's *t* test (*b*) $P<0.0001$, *c*) $P<0.001$).
d) Significantly different from the rats treated with DEN+IRG by Student's *t* test ($P<0.001$).
c) Significantly different from the rats treated with DEN+PB by Student's *t* test ($P<0.0001$).

genesis, together with an inverse correlation of the expression with hepatocellular proliferation, suggesting that Cx 32 expression has an important role in the hepatocarcinogenesis.[16)] The results of this study are consistent with such a hypothesis.

Eghbali *et al*.[26)] injected transfected and non-transfected human hepatoma cell lines into nude mice to examine the effect of the Cx 32 gene, and found that the tumor derived from Cx 32-transfected cells was significantly smaller than that derived from non-transfected cells. Negative growth control *in vivo* and *in vitro* was also observed in other tumorigenic cell lines transfected by gap junction genes.[27–31)] It is speculated that connexin gene expression basically increases gap junctional protein, enhances intercellular communication and influences cell proliferation. This may be one mechanism the suppressing effect of IRG on hepatocarcinogenesis.

Recently, IRG has been reported to inhibit the induction of tissue-type plasminogen activator synthesis in endothelial cells, and the angiogenesis induced by epidermal growth factor.[32)] The induction of tissue-type plasminogen activator is considered to be indispensable for growth factor-dependent angiogenesis.[33)] Larson and Haudenschild have reported that junctional coupling is slightly reduced during the repair of wounded aortic endothelial cells.[34)] On the other hand, Pepper and Meda indicated that migrating endothelial cells express plasminogen activator activity, while GJIC itself increases during the migration

of endothelial cells.[35] These findings suggest that IRG may suppress angiogenesis, which may also play an important role in tumor growth,[36] through abrogation of the induction of plasminogen activator by modulating GJIC of endothelial cells. This could be another mechanism by which IRG suppresses hepatocarcinogenesis. In addition, IRG has been shown to activate GJIC through the M_1 muscarinic acetylcholine receptor.[18] Activation of the M_1 receptor inhibits Raf activation induced by growth factors.[37] Therefore, the suppressive effect of IRG on hepatocarcinogenesis found in the present study may be due to signaling through the M_1 muscarinic acetylcholine receptor.

In this study, body weight gain tended to be decreased by the IRG treatment, though no particular toxic effects caused by IRG were found in histopathological examination. The dose of IRG in this study was the MTD value based on the previous study.[20] Further work is needed to confirm the chemopreventive effects at lower doses.

Although more studies are necessary to confirm dose-dependency, and to establish the precise mode of action of IRG and the underlying mechanisms during carcinogenesis, the results of the present investigation strongly suggest that IRG, currently being used as a therapeutic agent, could also be a promising chemopreventive agent for human liver neoplasia.

ACKNOWLEDGMENTS

We thank Dr. Kimihiko Satoh, Second Department of Biochemistry, Hirosaki University School of Medicine, for the gift of anti-GST-P antibody. We also wish to thank Ms. K. Takahashi and Mr. M. Ito for technical assistance. This work was supported in part by a Grant-in-Aid from the Ministry of Health and Welfare of Japan.

(Received October 20, 1997/Revised January 6, 1998/2nd Revised January 19, 1998/Accepted January 22, 1998)

REFERENCES

1) Wattenberg, L. W. Chemoprevention of cancer. *Cancer Res.*, **45**, 1–8 (1985).
2) Tanaka, T., Nishikawa, A., Shima, H., Sugie, S., Shinoda, T., Yoshimi, N., Iwata, H. and Mori, H. Inhibitory effects of chlorogenic acid, reserpine, polyprenoic acid (E-5166), or coffee on hepatocarcinogenesis in rats and hamsters. *In* "Antimutagenesis and Anticarcinogenesis Mechanisms," ed. Y. Kuroda, D. M. Shankel and M. D. Waters, pp. 429–440 (1990). Plenum Press, New York.
3) Salt, D. B. and Farber, E. New principle for the analysis of chemical carcinogenesis. *Nature*, **263**, 701–703 (1976).
4) Pitot, H. C., Barsness, L., Goldsworthy, T. and Kitagawa, T. Biochemical characterization of stages of hepatocarcinogenesis after a single dose of diethylnitrosamine. *Nature*, **271**, 456–458 (1978).
5) Ito, N., Tatematsu, M., Nakanishi, K., Hasegawa, R., Takano, T., Imaida, K. and Ogiso, T. The effects of various chemicals on the development of hyperplastic liver nodules in hepatectomized rats treated with N-nitrosodiethylamine or N-2-fluorenylacetamide. *Jpn. J. Cancer Res.*, **71**, 832–842 (1980).
6) Dragan, Y. P. and Pitot, H. C. The role of the stages of initiation and promotion in phenotypic diversity during hepatocarcinogenesis in the rat. *Carcinogenesis*, **13**, 739–750 (1992).
7) Loewenstein, W. R. Junctional intercellular communication and the control of growth. *Biochim. Biophys. Acta*, **560**, 1–65 (1979).
8) Yamasaki, H., Krutovskikh, V., Mesnil, M., Columbano, A., Tsuda, H. and Ito, N. Gap junction intercellular communication and cell proliferation during rat liver carcinogenesis. *Environ. Health Perspect.*, **101**, 191–198 (1993).
9) Tsuda, H., Asamoto, M., Baba, H., Iwahori, Y., Matsumoto, K., Iwase, Y., Nishida, Y., Nagao, S., Hakoi, K., Yamaguchi, S., Ozaki, K. and Yamasaki, H. Cell proliferation and advancement of hepatocarcinogenesis in the rat are associated with a decrease in connexin 32 expression. *Carcinogenesis*, **16**, 101–105 (1995).
10) Trosko, J. E. and Chang, C. C. Potential role of intercellular communication in the rate-limiting step in carcinogenesis. *J. Am. Coll. Toxicol.*, **2**, 5–22 (1983).
11) Saez, J. C., Conner, J. A., Spray, D. C. and Bennett, M. V. L. Hepatocyte gap junctions are permeable to a second messenger, inositol 1,4,5-triphosphate and to calcium ions. *Proc. Natl. Acad. Sci. USA*, **86**, 2708–2712 (1989).
12) Paul, D. L. Molecular cloning of cDNA for rat liver gap junction protein. *J. Cell Biol.*, **103**, 123–134 (1986).
13) Zhang, J.-T. and Nicholson, B. J. Sequence and tissue distribution of a second protein of hepatic gap junctions, Cx26, as deduced from its cDNA. *J. Cell Biol.*, **109**, 3391–3401 (1989).
14) Sakamoto, H., Oyamada, M., Enomoto, K. and Mori, M. Differential changes in expression of gap junction proteins connexin 26 and 32 during hepatocarcinogenesis in rats. *Jpn. J. Cancer Res.*, **83**, 1210–1215 (1992).
15) Sugie, S., Mori, H. and Takahashi, M. Effect of *in vivo* exposure to the liver tumor promoters phenobarbital or DDT on the gap junctions of rat hepatocytes; a quantitative freeze-fracture analysis. *Carcinogenesis*, **8**, 45–51 (1987).
16) Ueda, F., Kyoi, T., Mimura, K. and Yamamoto, M. Intercellular communication in cultured rabbit gastric epithelial cells. *Jpn. J. Pharmacol.*, **57**, 321–328 (1991).
17) Ueda, F., Kameda, Y., Yamamoto, O. and Shibata, Y. Beta-Adrenergic regulation of gap-junctional intercellular communication in cultured rabbit gastric epithelial cells. *J. Pharmacol. Exp. Ther.*, **271**, 397–402 (1994).

18) Ueda, F., Ban, K. and Ishima, T. Irsogradine activates gap-junctional intercellular communication through M_1 muscarinic acetylcholine receptor. *J. Pharmacol. Exp. Ther.*, **274**, 815–819 (1995).
19) Hosokawa, T., Ogawa, K., Otani, Y. and Kajiwara, T. Two cases of gastric cancer remarkably reduces with a combined dosage of irsogladine maleate preparation and UFT. *Oncol. Rep.*, **1**, 93–95 (1994).
20) Sumi, N., Yoshida, M., Nishiguchi, Y., Tawaratani, T., Fujimoto, S., Ishibashi, S., Yoshifusa, H., Kameyama, K. and Nomura, A. Twelve-months oral toxicity study of 2,4-diamino-6-(2,5-dichlorophenyl)-*s*-triazine maleate (MN-1695) in rats followed by two-months recovery test. *Pharmacometrics*, **32**, 347–386 (1986).
21) Sugie, S., Reddy, B. S., El-Bayoumy, K. and Tanaka, T. Inhibition by dietary benzylselenocyanate of hepatocarcinogenesis induced by azoxymethane in Fischer 344 rats. *Jpn. J. Cancer Res.*, **80**, 952–957 (1989).
22) Sato, K., Kitahara, A., Satoh, K., Ishikawa, T., Tatematsu, M. and Ito, N. The placental form of glutathione *S*-transferase as a new marker protein for preneoplasia in rat chemical hepatocarcinogenesis. *Gann*, **75**, 199–202 (1984).
23) Satoh, K., Kitahara, A., Soma, Y., Inaba, Y., Hayama, I. and Sato, K. Purification, induction and distribution of placental glutathione transferase: a new marker enzyme for preneoplastic cells in the rat chemical carcinogenesis. *Proc. Natl. Acad. Sci. USA*, **82**, 3964–3968 (1985).
24) Williams, G. M. The pathogenesis of rat liver cancer caused by chemical carcinogenesis. *Biochim. Biophys. Acta*, **605**, 167–189 (1980).
25) Stein, L. S., Boonstra, J. and Burghardt, R. C. Reduced cell-cell communication between mitotic and nonmitotic coupled cells. *Exp. Cell Res.*, **198**, 1–7 (1992).
26) Eghbali, B., Kessler, J. A., Reid, L. M., Roy, C. and Spray, D. C. Involvement of gap junctions in tumorigenesis: transfection of tumor cells with connexin 32 cDNA retards growth *in vivo*. *Proc. Natl. Acad. Sci. USA*, **88**, 10701–10705 (1991).
27) Naus, C. C. G., Elisevich, K., Zhu, D., Belliveau, D. J. and Del Maestro, R. F. *In vivo* growth of C6 glioma cells transfected with connexin 43 cDNA. *Cancer Res.*, **52**, 4208–4213 (1992).
28) Zhu, D., Caveney, S., Kidder, G. M. and Naus, C. C. G. Transfection of C6 glioma cells with connexin 43 cDNA: analysis of expression, intercellular coupling, and cell proliferation. *Proc. Natl. Acad. Sci. USA*, **88**, 1883–1887 (1991).
29) Mehta, P. P., Hotz-Wagenblatt, A., Rose, B., Shalloway, D. and Loewenstein, W. R. Incorporation of the gene for a cell-cell channel protein into transformed cells leads to normalization of growth. *J. Membr. Biol.*, **124**, 207–225 (1991).
30) Rose, B., Mehta, P. P. and Loewenstein, W. R. Gap-junction protein gene suppresses tumorigenicity. *Carcinogenesis*, **14**, 1073–1075 (1993).
31) Mesnil, M., Krutovskikh, V., Piccoli, C., Elfgang, C., Traub, O., Willecke, K. and Yamasaki, H. Negative growth control of HeLa cells by connexin genes: connexin species specificity. *Cancer Res.*, **55**, 629–639 (1995).
32) Sato, Y., Morimoto, A., Kiue, A., Okamura, K., Hamanaka, R., Kohno, K., Kuwano, M. and Sakata, T. Irsogladine is a potent inhibitor of angiogenesis. *FEBS Lett.*, **322**, 155–158 (1993).
33) Sato, Y., Okamura, K., Morimoto, A., Hamanaka, R., Hamaguchi, K., Shimada, T., Ono, M., Kohno, K., Sakata, T. and Kuwano, M. Indispensable role of tissue-type plasminogen activator in growth factor-dependent tube formation of human microvascular endothelial cells *in vitro*. *Exp. Cell Res.*, **204**, 223–229 (1993).
34) Larson, D. M. and Haudenschild, C. C. Junctional transfer in wounded cultures of bovine aortic endothelial cells. *Lab. Invest.*, **59**, 373–379 (1988).
35) Pepper, M. S. and Meda, P. Basic fibroblast growth factor increases junctional communication and connexin 43 expression in microvascular endothelial cells. *J. Cell. Physiol.*, **153**, 196–205 (1992).
36) Sipos, E. P., Tamargo, R. J., Weingart, J. D. and Brem, H. Inhibition of tumor angiogenesis. *Ann. NY Acad. Sci.*, **732**, 263–272 (1994).
37) Russel, M., Wintz, S. and Johnson, G. L. Acethylcholine muscarinic m1 receptor regulation of cyclic AMP synthesis controls growth factor stimulation of Raf activity. *Mol. Cell. Biol.*, **14**, 2343–2351 (1994).