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Chemopreventive Effect of N-(2-Cyclohexyloxy-4-nitrophenyl)methane Sulfonamide (NS-398), a Selective Cyclooxygenase-2 Inhibitor, in Rat Colon Carcinogenesis Induced by Azoxymethane

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Non-steroidal anti-inflammatory drugs (NSAIDs) such as sulindac and indomethacin inhibit colon carcinogenesis, and selective cyclooxygenase (COX)-2 inhibitors are considered to be potential chemopreventive agents without the side effects of usual NSAIDs. We reported that NS-398, N-(2-cyclohexyloxy-4-nitrophenyl)methane sulfonamide, suppressed the formation of preneoplastic lesions, aberrant crypt foci (ACF), induced by azoxymethane (AOM) in a short-term assay of rat colon carcinogenesis. In this study, we examined the effects of long-term NS-398 administration on rat colon carcinogenesis. After three AOM treatments at weekly intervals, a dose of 10 mg/kg of NS-398 in 5% Arabic gum solution was administered by gavage three times per week in group 2 until the termination of the experiment. Rats in group 1 were fed in a basal diet and given 5% Arabic gum solution alone after AOM treatment. At 40 weeks after the first AOM treatment, all rats were killed and the whole intestines including colon were examined. While the incidences of whole intestinal and colon neoplasms in group 1 were 84.6% and 80.8%, respectively, those in group 2 (given NS-398) were 51.9% and 44.4% respectively ($P=0.0177$ and $P=0.0103$ by Fisher's exact test, respectively). The multiplicities in group 2 (0.67 ± 0.78 and 0.48 ± 0.58) were also decreased significantly compared with those (1.39 ± 1.10 and 1.08 ± 0.74) in group 1 ($P<0.01$ by Welch's method and $P<0.002$ by Student's t test, respectively). In immunohistochemistry for proliferative cell nuclear antigen (PCNA), the PCNA-stained cell index (7.40 ± 0.5) in group 2 was significantly decreased from that in group 1 (14.03 ± 0.82) ($P<0.001$ by Welch's method). The results suggest that NS-398, a selective COX inhibitor, has a chemopreventive activity against colon carcinogenesis without side-effects such as gastric ulceration.

Key words: Cyclooxygenase-2 — Chemoprevention — Rat colon carcinogenesis

Colorectal cancer is one of the causes of increasing world-wide cancer mortality.[1)] Recently, emphasis has been placed on the identification of high-risk families and on appropriate surveillance regimens for normal and high-risk groups.[2, 3)] In parallel, studies have been done on the chemoprevention of colorectal cancer with NSAIDs.[4–6)] In addition, experimental models have provided means for examining cancer-preventive effects and obtaining insights into the early stages of tumor growth.[7–9)] Among the experimental models, there is the Min/+ mouse, which carries a fully penetrant dominant mutation of the murine *Apc* gene and develops adenomas throughout its intestinal tract, mostly in the small intestine, without carcinogen treatment, as is seen in human familial adenomatous polyposis. This model has also demonstrated the cancer-preventive potential of NSAIDs.[10, 11)]

At present, the mechanisms by which NSAIDs reduce colon carcinogenesis is considered to involve the inhibition of PG synthesis by inhibition of COX, which is one of the key enzymes in the arachidonic acid cascade to produce PGs.[12)] Two isozymes of COX, COX-1 and COX-2, have recently been identified.[13)] COX-2 is induced by some mitogens, cytokines and growth factors, and is responsible for production of PGs in inflammation.[14–16)] Furthermore, several researchers have reported that its expression is increased in human and animal colon cancers.[17–20)] It is, therefore, suggested that COX-2 expression is selectively increased during carcinogenesis and that its inhibition may account for the ability of NSAIDs to inhibit cancer development.

NS-398, synthesized by Taisho Pharmaceutical Co. (Tokyo), is a selective COX-2 inhibitor.[21)] Other selective COX-2 inhibitors, nimesulide and SC-58635, inhibited the development of ACF, putative preneoplastic lesions in rat

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Abbreviations: NSAIDs, non-steroidal anti-inflammatory drugs; PG, prostaglandin; COX, cyclooxygenase; ACF, aberrant crypt foci; AOM, azoxymethane; PCNA, proliferative cell nuclear antigen; b.w., body weight.

colon carcinogenesis, in rats treated with the carcinogen AOM.[22, 23] We have also reported similar results with NS-398.[24] In this study, we examined the chemopreventive potential of NS-398 in rat colon carcinogenesis using a long-term assay.

MATERIALS AND METHODS

Chemicals AOM was purchased from Sigma (St. Louis, MO) as the carcinogen for colon carcinogenesis. NS-398, N-(2-cyclohexyloxy-4-nitrophenyl)methane sulfonamide, was supplied by Taisho Pharmaceutical Co. through Dr. N. Futaki (Research Center, Taisho Pharmaceutical Co., Saitama). The molecular structure of NS-398 is shown in Fig. 1.

Animal treatment We used two different protocols. The experimental designs are shown in Fig. 2. Eighty-three male F344 rats, 4 weeks old, purchased from Japan SLC Inc. (Hamamatsu) were used. The rats were divided into four groups (Table I), and kept in a room controlled at 23±2°C, 50%±10% humidity and a 12 h light/dark cycle. Groups 1 and 2 were treated with AOM, 15 mg/kg b.w., s.c., at 6, 7 and 8 weeks of age. Groups 2 and 3 were administered NS-398, 10 mg/kg b.w., in 5% Arabic gum solvent, by oral gavage, 3 times (Monday, Wednesday and Friday) per week during the experiment. Groups 1 and 4 were treated with only 5% Arabic gum without NS-398. At 40 weeks after first AOM treatment, all rats were killed and the entire intestines from duodenum to anus were removed, opened and flushed with saline. Neoplasms were counted in the entire intestine, including the colon. Each neoplasm was measured and the size was calculated as $4\pi/3\times$(the product of the lengths along three perpendicular axes). Then each colon was fixed flat on a paper filter in 10% buffered formalin to observe ACF. All neoplasms were removed. The neoplasms and remaining intestines were embedded in paraffin for histological examination according to the criteria of Ward.[25]

Identification of ACF The fixed colons were stained with 0.5% methylene blue in saline. ACF were recorded according to the procedure of Bird[26] and our laboratory,[24] but some colons with large neoplasms were omitted, because the number of ACF in the whole colon could not be counted accurately. ACF were distinguished from the

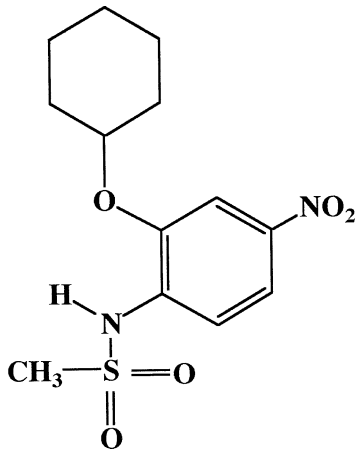

Fig. 1. The chemical structure of NS-398, N-(2-cyclohexyloxy-4-nitrophenyl)methane sulfonamide.

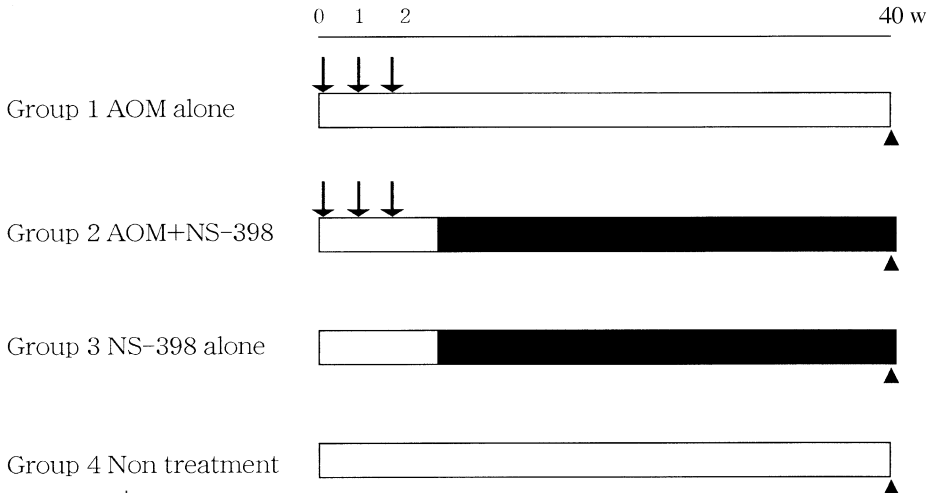

Fig. 2. Experimental protocol. ↓ AOM, 15mg/kg, s.c. injection; ▲ killed; ▭ basal diet (CE-2) and 5% Arabic gum aqueous solution by oral gavage, 3 times a week; ■ basal diet and NS-398, 10 mg/kg in 5% Arabic gum aqueous solution by oral gavage, 3 times a week.

surrounding normal crypts by their swollen character and discernible pericryptal zone. The occurrence and the multiplicity of ACF were assessed. The crypt multiplicity means the number of aberrant crypts in each focus and foci were categorized as those containing four to ten, and those containing eleven or more aberrant crypts/focus. The scores were checked by two observers in a double-blinded fashion.

PCNA immunohistochemistry The colonic mucosa (except neoplasms) was used for PCNA immunohistochemical analysis after observation of ACFs. The immunohistochemical stainings were performed according to the methods in our previous paper.[27] The embedded tissues were cut into 4 μm sections, then stained by using PCNA antibody (Novocastra Lab., Newcastle, UK) and an ABC kit (Vector Lab., Burlingame, CA). The number of PCNA-positive nuclei in crypts per section was counted as described in previous papers.[27, 28]

Statistical analysis Data obtained in this study are presented as mean±SD, and Student's *t* test, Welch's method or Fisher's exact test was used to determine the significance of differences between groups. Differences were considered to be significant at the $P<0.05$ level.

RESULTS

There were no differences of body weight, liver weight and the relative ratio of liver weight to body weight among groups at the experimental termination (Table I). Histopathologically, no differences were seen between groups treated with and without NS-398. In this study, no stomachs had erosive or ulcerative changes, and there were no toxic changes in the liver and kidney.

The incidence and multiplicity of the neoplasms in each group are summarized in Table II. While the incidences of neoplasms in small intestine, colon, and entire intestine of group 1 treated with AOM alone were 26.9%, 80.8% and 84.6%, respectively, those in group 2 given both AOM and NS-398 were 14.8%, 44.4% and 51.9%, respectively. There were significant differences in colon and total incidences between these groups ($P=0.0103$ and $P=0.0177$ by Fisher's exact test, respectively). The multiplicities of the neoplasms in colon, and entire intestine of group 2 (0.48±0.58 and 0.67±0.78, respectively) also were significantly lower than those of group 1 (1.08±0.74 and 1.39±1.10, respectively) ($P<0.002$ by Student's *t* test and $P<0.01$ by Welch's method, respectively). Histopathologically, 17 adenomas and 19 adenocarcinomas in group 1 and 10 adenomas and 8 adenocarcinomas in group 2 were induced under overall observation, and there were no histological differences even in the small intestine and colon. The sizes of neoplasms in both groups were not different (1053±1525 and 986±1715 mm^3, respectively).

The ACF counts at 40 weeks after AOM treatment in groups 1 and 2 are summarized in Table III. Total numbers of ACF per colon, the number of ACF containing 4–

Table I. Body Weight, Liver Weight and the Relative Ratio of Liver Weight to Body Weight at Experimental Termination

Group	Treatment	No. of rats	Body weight (g)	Liver weight (g)	Relative ratio (L/B×100)
1	AOM alone	26	326±32	11.1±1.4	3.4±0.4
2	AOM+10 mg/kg NS-398	27	314±30	10.8±1.9	3.4±0.6
3	10 mg/kg NS-398 alone	10	343±25	11.5±1.5	3.3±0.5
4	Non treatment	10	338±32	11.2±1.5	3.3±0.2

There were no significant differences between any of the groups.

Table II. Incidence and Multiplicity of Intestinal Neoplasms in Each Group

Group	Treatment	No. of rats	Incidence (%)			Multiplicity		
			Small intestine	Colon	Entire intestine	Small intestine	Colon	Entire intestine
1	AOM alone	26	7 (26.9)	21 (80.8)	22 (84.6)	0.31±0.55	1.08±0.74	1.39±1.10
2	AOM+10 mg/kg NS-398	27	4 (14.8)	12 (44.4)[a]	14 (51.9)[b]	0.19±0.48	0.48±0.58[c]	0.67±0.78[d]
3	10 mg/kg NS-398 alone	10	0	0	0	0	0	0
4	Non treatment	10	0	0	0	0	0	0

a) Significantly different from group 1 by Fisher's exact test ($P=0.0103$).
b) Significantly different from group 1 by Fisher's exact test ($P=0.0177$).
c) Significantly different from group 1 by Student's *t* test ($P<0.002$).
d) Significantly different from group 1 by Welch's method ($P<0.01$).

Table III. Occurrence of AOM-induced ACF Formation at Experimental Termination

Group	Treatment	No. of examined rats	Total number of ACF/colon	No. of foci containing 4–10 crypts	No. of foci containing 11 or more crypts
1	AOM alone	17	115.4±54.2	45.1±23.4	5.65±6.50
2	AOM→10 mg/kg NS-398	21	84.3±38.4[a)]	30.3±15.7[b)]	1.52±1.81[c)]

a) Significant difference from group 1 by Student's *t* test ($P<0.05$).
b, *c*) Significant difference from group 1 by Welch's method ($P<0.05$ and $P<0.02$, respectively).

Table IV. PCNA-staining Index of Colonic Crypts in Each Group

Group	Treatment	No. of rats	PCNA-staining index (%)
1	AOM alone	26	14.03±0.82[a)]
2	AOM+10 mg/kg NS-398	27	7.40±0.56[b,c)]
3	10 mg/kg NS-398 alone	10	6.27±2.09
4	Non treatment	10	5.97±1.92

a) Significantly different from group 4 by Welch's method ($P<0.001$).
b) Significantly different from group 1 by Welch's method ($P<0.001$).
c) Significantly different from group 4 by Welch's method ($P<0.05$).

10 crypts and the number of ACF containing 11 or more crypts in group 2 (84.3±38.4, 30.3±15.7 and 1.52±1.81, respectively) were significantly reduced as compared with those in group 1 (115.4±54.2, 45.1±23.4 and 5.65±6.50, respectively) ($P<0.05$ by Student's *t* test and $P<0.05$ and $P<0.02$ by Welch's method, respectively).

In immunohistochemistry for PCNA, the appearance of PCNA-stained cells in groups 1 and 2 was increased by the exposure to AOM, compared with that in group 4 (Table IV). However, the PCNA-stained cell index (7.40±0.5) in group 2 was significantly decreased from that in group 1 (14.03±0.82) ($P<0.001$ by Welch's method). It is suggested that NS-398 inhibited the cell proliferation induced by AOM.

DISCUSSION

Although NSAIDs are highly effective for the relief of inflammatory disease as COX inhibitors, their use is limited by adverse effects, particularly on the gastrointestinal tract and kidneys. COX is one of the rate-limiting enzymes in PG synthesis,[12)] and two isozymes, constitutive COX-1 and inducible COX-2, are known.[13)] Since COX-1 exists universally in most tissues and is involved in the physiological function of PGs under normal homeostasis,[12, 29)] the adverse effects are considered to be related to the inhibition of the physiological expression of COX-1 in stomach and kidney. Therefore, selective COX-2 inhibitors have been sought.[21)] NS-398 is one of these, and has been reported to be a strongly selective COX-2 inhibitor.[30–32)] It produced little gastric ulceration and did not damage the duodenum of rats.[33–35)]

In the previous paper, we reported the inhibitory effect of NS-398 on the development of ACF in rat colon carcinogenesis[24)] as well as the results for other selective COX-2 inhibitors.[22, 23)] In this study, we confirmed the chemopreventive effect in rat colon carcinogenesis. Reddy *et al*. have already reported the chemopreventive activity of SC-58635, celecoxib, in rat colon carcinogenesis.[36)] They observed powerful inhibition of tumorigenicity in the colon (93% inhibition). They used 1500 ppm of the chemical in diet, while we administered 10 mg/kg of NS-398 by gavage, 3 times a week. Other researchers have reported the chemopreventive activity of another selective COX-2 inhibitor, nimesulide, against tumorigenicity in mouse intestines and rat urinary bladder.[37–39)]

The results, including our data, suggest that tumorigenicity in the intestinal tract is related to the overexpression of COX-2, since this isozyme is known to be overexpressed in human and animal neoplasms.[17–20)] In fact, Oshima *et al*. showed that COX-2 inactivation suppressed polyp growth rather than polyp initiation by using *COX-2* gene knockout mice containing a mutated *Apc* gene.[40)] Therefore, the inhibition of PG synthesis appears to be an important component of the mechanism of action of selective COX-2 inhibitors, including NS-398. In addition to the inhibition of PG synthesis, since the overexpression of COX-2 in rat intestinal epithelial cells induces a G1 delay and renders the cells resistant to the induction of apoptosis by sodium butyrate,[41, 42)] the inhibitory effects of NS-398 may be related to restoration of normal apoptotic mechanisms. We have already observed the apoptotic potential of NS-398 in human colon cancer cultured cells,[43)] and other reports have noted similar results.[44, 45)]

In this study, we found that the increase of ACF containing 11 and more crypts, which were considered as preneoplastic lesion, was significantly inhibited in rats treated with NS-398. The findings may be related to the induction of apoptosis in addition to the inhibition of cell growth by NS-398. Furthermore, it has recently been reported that cyclooxygenase regulates angiogenesis in colon cancer cell lines.[46] COX-2 modulated the production of angiogenic factors by colon cancer cells, while COX-1 regulated angiogenesis in endothelial cells, suggesting a possible mechanism whereby COX-2 inhibitors inhibit tumor growth. In conclusion, we have shown that NS-398, a selective inhibitor, has potential as a chemopreventive agent in colon carcinogenesis. Selective COX-2 inhibitors may be effective in colorectal cancer prevention, because these agents are likely to be less toxic than usual NSAIDs, including aspirin and sulindac. However, since it has been suggested that NSAIDs inhibit colon carcinogenesis via COX-independent mechanisms,[47, 48] further studies on the mechanisms involved are needed.

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REFERENCES

1) Broder, S. Perspectives on cancer in Japan and the United States. *Jpn. J. Cancer Res.*, **83**, 821–830 (1993).
2) Sandler, R. S., DeVellis, B. M., Blalock, S. J. and Holland, K. L. Participation of high-risk subjects in colon cancer screening. *Cancer*, **63**, 2211–2215 (1989).
3) Guillem, J. G., Forde, K. A., Treat, M. R., Neugut, A. I., O'Toole, K. M. and Diamond, B. E. Colonoscopic screening for neoplasms in asymptomatic first-degree relatives of colon cancer patients. A controlled, prospective study. *Dis. Colon Rectum*, **35**, 523–529 (1992).
4) Waddell, W. R. and Loughry, R. W. Sulindac for polyposis of the colon. *J. Surg. Oncol.*, **24**, 83–87 (1983).
5) Thun, M. J., Namboodiri, M. N. and Heath, C. W. Aspirin use and reduced risk of fatal colon cancer. *N. Engl. J. Med.*, **325**, 1593–1596 (1991).
6) Gann, P. H., Manson, J. E., Glynn, R. J., Buring, J. E. and Hennekens, C. H. Low dose aspirin and incidence of colorectal tumours in a randomized trial. *J. Natl. Cancer Inst.*, **85**, 1220–1224 (1993).
7) Tanaka, T., Kojima, T., Yoshimi, N., Sugie, S. and Mori, H. Inhibitory effect of the non-steroidal anti-inflammatory drug, indomethacin, on the naturally occurring carcinogen, 1-hydroxyanthraquinone in male ACI/N rats. *Carcinogenesis*, **12**, 1949–1952 (1991).
8) Reddy, B. S., Rao, C. V., Rivenson, A. and Kelloff, G. Inhibitory effect of aspirin on azoxymethane-induced colon carcinogenesis in F344 rats. *Carcinogenesis*, **14**, 1493–1497 (1993).
9) Rao, C. V., Rivenson, A., Simi, B., Zang, E., Kelloff, G., Steele, V. and Reddy, B. S. Chemoprevention of colon carcinogenesis by sulindac, a nonsteroidal anti-inflammatory agent. *Cancer Res.*, **55**, 1464–1472 (1995).
10) Beazer-Barclay, Y., Levy, D. B., Moser, A. R., Dove, W. F., Hamilton, S. R., Volgelstein, B. and Kinzler, K. W. Sulindac suppresses tumorigenesis in the Min mouse. *Carcinogenesis*, **17**, 1757–1760 (1996).
11) Jacoby, R. F., Marshall, D. J., Newton, M. A., Novakovic, K., Tutsch, K., Cole, C. E., Lubet, R. A., Kellof, G. J., Verma, A., Moser, A. R. and Dove, W. F. Chemoprevention of spontaneous intestinal adenomas in the Apc Min mouse model by the non-steroidal anti-inflammatory drug piroxicam. *Cancer Res.*, **56**, 710–714 (1996).
12) Smith, W. L. and Marnett, L. Prostaglandin endoperoxide synthase: structure and catalysis. *Biochim. Biophys. Acta*, **1083**, 1–17 (1991).
13) Feng, L., Sun, W., Xia, Y., Tang, W. W., Chanmugam, P., Soyoola, E., Wilson, C. B. and Hwang, D. Cloning two isoforms of rat cyclooxygenase: differential regulation of their expression. *Arch. Biochem. Biophys.*, **307**, 361–368 (1993).
14) Kujubu, D. A., Fletcher, B. S., Varnum, B. C., Lim, R. W. and Herschman, H. R. TIS10, a phorbol ester tumor promoter-inducible mRNA from Swiss 3T3 cells, encodes a novel prostaglandin synthase/cyclooxygenase homologue. *J. Biol. Chem.*, **266**, 12866–12872 (1991).
15) Hla, T. and Neilson, K. Human cyclooxygenase-2 cDNA. *Proc. Natl. Acad. Sci. USA*, **89**, 7384–7388 (1992).
16) Hla, T., Kistimaki, A., Appleby, S. and Barriocanel, J. G. Cyclooxygenase gene expression in inflammation and angiogenesis. *Ann. NY Acad. Sci.*, **696**, 197–204 (1993).
17) Eberhart, C. E., Coffey, R. J., Radhika, A., Giardiello, F. M., Ferrenbach, S. and DuBois, R. N. Up-regulation of cyclooxygenase-2 gene expression in human colorectal adenomas and adenocarcinomas. *Gastroenterology*, **107**, 1183–1188 (1994).
18) Sano, H., Kawahito, Y., Wilder, R.L., Hashiramoto, A., Mukai, S., Asai, K., Kimura, S., Kato, H., Kondo, M. and Hla, T. Expression of cyclooxygenase-1 and -2 in human colorectal cancer. *Cancer Res.*, **55**, 3785–3789 (1995).
19) Yoshimi, N., Ino, N., Suzui, M., Tanaka, T., Nakashima,

S., Nakamura, M., Nozawa, Y. and Mori, H. The mRNA overexpression of inflammatory enzymes, phospholipase A2 and cyclooxygenase, in the large bowel mucosa and neoplasms of F344 rats treated with naturally occurring carcinogen, 1-hydroxyanthraquinone. *Cancer Lett.*, **97**, 75–82 (1995).

20) DuBois, R. N., Radhika, A., Reddy, B. S. and Entingh, A. J. Increased cyclooxygenase-2 levels in carcinogen-induced rat clonic tumors. *Gastroenterology*, **110**, 1259–1262 (1996).
21) Vane, J. R. and Botting, R. M. Overview—mechanisms of action of anti-inflammatory drugs. *In* "Improved Non-steroidal Anti-inflammatory Drugs: COX-2 Enzyme Inhibitors," ed. J. Vane, J. Botting, and R. Botting, pp. 1–27 (1996). Kluwer Academic Publishers, Dordrecht.
22) Takahashi, M., Fukutake, M., Yokota, S., Ishida, K., Wakabayashi, K. and Sugimura, T. Suppression of azoxymethane-induced aberrant crypt foci in rat colon by nimesulide, a selective inhibitor of cyclo-oxygenase-2. *J. Cancer Res. Clin. Oncol.*, **122**, 733–737 (1996).
23) Reddy, B. S., Rao, C. V. and Seibert, K. Evaluation of cyclooxygenase-2 inhibitor for potential chemopreventive properties in colon carcinogenesis,. *Cancer Res.*, **56**, 4566–4569 (1996).
24) Yoshimi, N., Kawabata, K., Hara, A., Matsunaga, K., Yamada, Y. and Mori, H. Inhibitory effect of NS-398, a selective cyclooxygenase-2 inhibitor, on azoxymethane-induced aberrant crypt foci in colon carcinogenesis of F344 rats. *Jpn. J. Cancer Res.*, **88**, 1044–1051 (1997).
25) Ward, J. M. Morphogenesis of chemically induced neoplasms of the colon and small intestine in rats. *Lab. Invest.*, **30**, 505–513 (1974).
26) Bird, R. P. Observation and quantification of aberrant crypts in the murine colon treated with a colon carcinogen: preliminary findings. *Cancer Lett.*, **37**, 147–151 (1987).
27) Wang, A., Yoshimi, N., Tanaka, T. and Mori, H. Inhibitory effects of magnesium hydroxide on c-myc expression and cell proliferation induced by methylazoxymethanol acetate in rat colon. *Cancer Lett.*, **75**, 73–78 (1993).
28) Mori, H., Mori, Y., Tanaka, T., Yoshimi, N., Sugie, S., Kawamori, T. and Narisawa, T. Cell kinetic analysis of the mucosal epithelium and assay of ornithine decarboxylase activity during the process of 1-hydroxyanthraquinone-induced large bowel carcinogenesis in rats. *Carcinogenesis*, **13**, 2217–2220 (1992).
29) Dewitt, D. L., Day, J., Sonnenberg, W. K. and Smith, W. L. Concentration of prostaglandin endoperoxide synthase and prostaglandin I2 synthase in the endothelium and smooth muscle of bovine aorta. *J. Clin. Invest.*, **72**, 1882–1888 (1983).
30) Futaki, N., Arai, I., Hamasaka, Y., Takahashi, S., Higuchi, S. and Otomo, S. Selective inhibition of NS-398 on prostanoid production in inflamed tissue in rat-carrageenan-air-pouch inflammation. *J. Pharm. Pharmacol.*, **45**, 753–755 (1993).
31) Futaki, N., Takahashi, S., Yokoyama, M., Arai, I., Higuchi, S. and Otomo, S. NS-398, a new anti-inflammatory agent, selectively inhibits prostaglandin G/H synthase/cyclooxygenase (COX-2) activity *in vitro*. *Prostaglandins*, **47**, 55–59 (1994).
32) Huff, R., Collins, P., Kramer, S., Seibert, K., Koboldt, C., Gregory, S. and Isakson, P. A structural feature of N-[2-(cyclohexyloxy)-4-nitrophenyl]methanesulfonamide (NS-398) that governs its selectivity and affinity for cyclooxygenase 2 (COX2). *Inflamm. Res.*, **44** (Suppl. 2), S145–S146 (1994).
33) Futaki, N., Yoshikawa, K., Hamasaka, Y., Arai, I., Higuchi, S., Iizuka, H. and Otomo, S. NS-398, a novel non-steroidal anti-inflammatory drug with potent analgesic and antipyretic effects, which causes minimal stomach lesions. *Gen. Pharmacol.*, **24**, 105–110 (1993).
34) Arai, I., Hamasaka, Y., Futaki, N., Takahashi, S., Yoshikawa, K., Higuchi, S. and Otomo, S. Effect of NS-398, a new nonsteroidal anti-inflammatory agent, on gastric ulceration and acid secretion in rats. *Res. Commun. Chem. Pathol. Pharmacol.*, **81**, 259–270 (1993).
35) Hirata, T., Ukawa, H., Kitamura, M. and Takeuchi, K. Effects of selective cyclooxygenase-2 inhibitors on alkaline secretory and mucosal ulcerogenic responses in rat duodenum. *Life Sci.*, **61**, 1603–1611 (1997).
36) Kawamori, T., Rao, C. V., Seibert, K. and Reddy, B. S. Chemopreventive activity of celecoxib, a specific cyclooxygenase-2 inhibitor, against colon carcinogenesis. *Cancer Res.*, **58**, 409–412 (1998).
37) Nakatsugi, S., Fukutake, M., Takahashi, M., Fukuda, K., Isoi, T., Taniguchi, Y., Sugimura, T. and Wakabayashi, K. Suppression of intestinal polyp development by nimesulide, a selective cyclooxygenase-2 inhibitor, in Min mice. *Jpn. J. Cancer Res.*, **88**, 1117–1120 (1997).
38) Fukutake, M., Nakatsugi, S., Isoi, T., Takahashi, M., Ohta, T., Mamiya, S., Taniguchi, Y., Sato, H., Fukuda, K., Sugimura, T. and Wakabayashi, K. Suppressive effects of nimesulide, a selective inhibitor of cyclooxygenase-2, on azoxymethane-induced colon carcinogenesis in mice. *Carcinogenesis*, **19**, 1939–1942 (1998).
39) Okajima, E., Denda, A., Ozono, S., Takahama, M., Akai, H., Sasaki, Y., Kitayama, W., Wakabayashi, K. and Konishi, Y. Chemopreventive effects of nimesulide, a selective cyclooxygenase-2 inhibitor, on the development of rat urinary bladder carcinomas initiated by N-butyl-N-(4-hydroxybutyl)nitrosamine. *Cancer Res.*, **58**, 3028–3031 (1998).
40) Oshima, M., Dinchuk, J. E., Kargman, S., Oshima, H., Hancock, B., Kwong, E., Trzaskos, J. M., Evans, J. F. and Taketo, M. M. Suppression of intestinal polyps in Apc D761 knockout mice by inhibition of cyclooxygenase-2 (COX-2). *Cell*, **87**, 803–809 (1996).
41) Tsujii, M. and DeBois, R. N. Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase-2. *Cell*, **83**, 493–501 (1995).
42) DuBois, R. N., Shao, J. Y., Tsujii, M., Sheng, H. M. and

Beauchamp, R. D. G1 delay in cells overexpressing prostaglandin endoperoxide synthase-2. *Cancer Res.*, **56**, 733–737 (1996).

43) Hara, A., Yoshimi, N., Niwa, M., Ino, N. and Mori, H. Apoptosis induced by NS-398, a selective cyclooxygenase-2 inhibitor, in human colorectal cancer cell lines. *Jpn. J. Cancer Res.*, **88**, 600–604 (1997).

44) Sawaoka, H., Kawano, S., Tsuji, S., Tsujii, M., Gunawan, E. S., Takei, Y., Nagano, K. and Mori, M. Cyclooxygenase-2 inhibitors suppress the growth of gastric cancer xenografts via induction of apoptosis in nude mice. *Am. J. Physiol.*, **274**, G1061–G1067 (1998).

45) Lin, X.-H., Yao, S., Kirschenbaum, A. and Levine, A. C. NS398, a selective cyclooxygenase-2 inhibitor, induces apoptosis and down-regulates bcl-2 expression in LNCaP cells. *Cancer Res.*, **58**, 4245–4249 (1998).

46) Tsujii, M., Kawano, S., Tsuji, S., Sawaoka, H., Hori, M. and DuBois, B. N. Cyclooxygenase regulates angiogenesis induced by colon cancer cells. *Cell*, **93**, 705–716 (1998).

47) Elder, D. J. E., Hague, A., Hicks, D. J. and Paraskeva, C. Differential growth inhibition by the aspirin metabolite salicylate in human colorectal tumor cell lines: enhanced apoptosis in carcinoma and *in vitro* transformed adenoma relative to adenoma cell lines. *Cancer Res.*, **56**, 2273–2276 (1996).

48) Hanif, R., Pittas, A., Feng, Y., Koutsos, M. I., Qiao, L., Staino-Coico, L., Shiff, S. I. and Rigas, B. Effects of nonsteroidal anti-inflammatory drugs on proliferation and on induction of apoptosis in colon cancer cells by a prostaglandin independent pathway. *Biochem. Pharmacol.*, **52**, 237–245 (1996).