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# A lipophilic statin, pitavastatin, suppresses inflammation-associated mouse colon carcinogenesis

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**3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors are known to modulate carcinogenesis. In this study, we investigated whether a lipophilic HMG-CoA reductase inhibitor pitavastatin suppresses inflammation-related mouse colon carcinogenesis. Male CD-1 (ICR) mice were initiated with a single intraperitoneal injection of azoxymethane (AOM, 10 mg/kg body weight) and promoted by 2% (w/v) dextran sodium sulfate (DSS) in drinking water for 7 days. The experimental diets containing pitavastatin at 2 dose levels (1 and 10 ppm) were fed to male CD-1 (ICR) mice for 17 weeks, staring 1 week after the cessation of DSS exposure. The effects of dietary pitavastatin on colonic tumor development were assessed at Weeks 5, 10 and 20. Feeding with pitavastatin at both doses significantly inhibited the multiplicity of colonic adenocarcinoma at Week 20. Furthermore, the treatment significantly lowered the positive rates of proliferating cell nuclear antigen and increased the apoptotic index in the colonic epithelial malignancies. The treatment also reduced nitrotyrosine-positivity in the colonic mucosa. Our findings thus show that pitavastatin is effective in inhibiting colitis-related colon carcinogenesis through modulation of mucosal inflammation, oxidative/nitrosative stress, and cell proliferation.**


**Key words:** statin; chemoprevention; inflammation; colon carcinogenesis; mouse

Statins, which are 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, are commonly-used drugs for the treatment of hypercholesterolemia.[1,2] They are able to decrease low-density lipoprotein (LDL) cholesterol levels by inhibiting HMG-CoA reductase. Furthermore, a triglyceride (TG)-lowering effect and a high-density lipoprotein (HDL) cholesterol-raising effect were observed in patients with hyperlipidemia, who take statins.[3,4] Statins have multibiological effects other than antilipidemia. Recently, it has been highlighted that statins are linked with several beneficial effects beyond their effect on cardiovascular disease. They include reduction in the risk of dementia,[5,6] fracture[7] and cancer.[8–10] Several recent preclinical studies indicated that statins may have chemopreventive potential against cancer at various sites,[10–12] including colon.[13–16] In addition, there is growing evidence that statins exert anti-inflammatory and antioxidative actions that are independent of their serum lipid lowering effects.[17]

Association between inflammation and cancer has long been suspected.[18,19] An example is that inflamed colon is a high risk for colorectal cancer (CRC) development.[20] CRC is thus one of the most serious complications of inflammatory bowel disease (IBD), including ulcerative colitis (UC)[20] and Crohn's disease (CD).[21] For understanding the pathogenesis of IBD and IBD-related CRC, we have developed a novel colitis-related and two-stage mouse CRC model, using a colon carcinogen azoxymethane (AOM) and a colitis-inducing agent dextran sodium sulfate (DSS).[22] In this animal model, numerous large bowel adenocarcinomas occur within a short-term period, and their histology and biological alterations resemble those found in human.[22] The model can be used for investigating and determining cancer chemopreventive agents against CRC[23] as well as initiating or modulating agents for CRC.[24]

A lipophilic statin pitavastatin, (+)-monocalcium bis{(3*R*,5*S*,6*E*)-7-[2-cyclopropyl-4-(4-fluorophenyl)-3-quinolyl]-3,5-dihydroxy-6-heptenoate} ($C_{50}H_{46}CaF_2N_2O_8$, MW 880.98, Fig. 1), that has been developed in Japan is highly effective for lowering serum cholesterol and TG levels.[25] The lowering effect of pitavastatin on serum LDL-cholesterol is more potent than that of pravastatin, simbastatin and atorvastatin.[26–28] The drug possessing a high oral bioavailability is only slightly metabolized, suggesting a longer duration of action and is less potent for drug interactions.[28] Therefore, the agent is currently undergoing Phase III trials in Europe, US and Japan.[25] Since pitavastatin possesses pleiotrophic biological effects, including anti-inflammatory actions,[29,30] we in the present study investigated the potential chemopreventive ability of colitis-related colon cancer development using our mouse model[22] to find desirable cancer chemopreventers against IBD-related CRC.[31] Since numerous evidence demonstrates that a high-fat diet is associated with the risk of CRC development and serum levels of TG and cholesterol are positively associated with colon carcinogenesis,[32] we monitored serum levels of TG and cholesterol during the study.

## Material and methods

### *Animals, chemicals and diets*

Male Crj: CD-1 (ICR) mice (Charles River Japan, Tokyo, Japan) aged 5 weeks were used in this study. They were maintained at Kanazawa Medical University Animal Facility according to the Institutional Animal Care Guideline. All animals were housed in plastic cages (4 or 5 mice/cages) with free access to drinking water and pelleted basal diet, CRF-1 (Oriental Yeast, Tokyo, Japan), under controlled conditions of humidity (50 ± 10%), light (12/12 hr light/dark cycle) and temperature (23 ± 2°C). After arrived, animals were quarantined for the first 7 days, and then randomized by their body weights into experimental and control groups. A colonic carcinogen AOM was purchased from Sigma Chemical (St. Louis, MO). DSS with a molecular weight of 36000–50000 (Cat. No. 160110) was purchased from MP Biomed-

*Abbreviations:* AOM, azoxymethane; CD, Crohn's disease; CRC, colorectal cancer; DSS, dextran sodium sulfate; H&E, hematoxylin and eosin; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; IBD, inflammatory bowel disease; iNOS, inducible bitric oxide synthase; LDL, low-density lipoprotein; NF-κB, nuclear factor-kappa B; NO, nitric oxide; PCNA, proliferating cell nuclear antigen; PSC, primary sclerosing cholangitis; ssDNA, single-stranded DNA; TG, triglycerides; UC, ulcerative colitis; UDCA, ursodeoxycholic acid.

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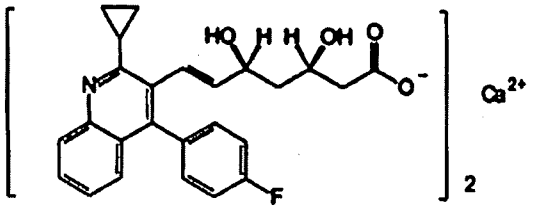


FIGURE 1 – Chemical structure of pitavastatin. (+)-Monocalcium bis[(3*R*,5*S*,6*E*)-7-[2-cyclopropyl-4-(4-fluorophenyl)-3-quinolyl]-3,5-dihydroxy-6-heptenoate]. $C_{50}H_{46}CaF_2N_2O_8$, MW 880.98.

icals, LLC (Aurora, OH). DSS for induction of colitis was dissolved in water at a concentration of 2% (w/v).

*Experimental procedures*

A total of 132 male ICR mice were divided into 7 experimental and control groups (Fig. 2). Mice in Groups 1–3 were given a single intraperitoneal injection of AOM (10 mg/kg body weight). Starting 1 week after the injection, animals received 2% DSS in the drinking water for 7 days. Subsequently, they were fed the diets containing 0, 1 and 10 ppm pitavastatin for 17 weeks, respectively, starting 1 week after the cessation of DSS exposure. Group 4 was fed the diet containing 10 ppm pitavastatin, and received no further treatments. Groups 5 and 6 were given AOM alone and DSS alone, respectively. Group 7 was an untreated control. Animals are sequentially sacrificed at Weeks 5, 10 and 20 by ether overdose to determine the effects of pitavastatin on colon tumorigenesis and biochemical profiles, including serum lipids measurements. Prior to sacrifice, animals were starved overnight for clinical chemistry. At sacrifice, the large bowels were flushed with saline, and excised. After measuring their length (from the ileocecal junction to the anal verge), large bowels were cut open longitudinally along the main axis, and gently washed with saline. The whole large bowel was macroscopically inspected for the presence of tumors, cut along a vertical axis and fixed in 10% buffered formalin for a least 24 hr. Histopathological examination was performed on paraffin-embedded sections after hematoxylin and eosin (H&E) staining. On H&E-stained sections, pathological lesions, such as mucosal ulceration, dysplasia and colonic tumors, were determined.

*Clinical chemistry*

At autopsy, whole blood anticoagulated with heparin lithium was taken from the inferior vena cava with a sterile syringe (Terumo, Tokyo, Japan) at each time point. The serum was obtained by centrifugation (3,000 rpm for 10 min), and stored at −80°C until measurement. Serum cholesterol was determined enzymatically using cholesterol esterase and cholesterol oxidase. The serum TG was assayed by enzymatic hydrolysis with lipase. These measurements were expressed as mg/dL.

*Scoring of inflammation in the large bowel*

Inflammation in the large bowel was scored on the H&E-stained sections. For scoring, large intestinal inflammation was graded according to the following morphological criteria described by Cooper *et al.*[33]: Grade 0, normal appearance; Grade 1, shortening and loss of the basal 1/3 of the actual crypts with mild inflammation in the mucosa; Grade 2, loss of the basal 2/3 of the crypts with moderate inflammation in the mucosa; Grade 3, loss of the entire crypts with severe inflammation in the mucosa and submucosa, but with retainment of the surface epithelium; Grade 4, presence of mucosal ulcer with severe inflammation (infiltration of neutrophils, lymphocytes, and plasma cells) in the mucosa, submucosa, muscularis propria and/or subserosa. The scoring was made on the entire colon with or without proliferative lesions and expressed as a mean average score/mouse.

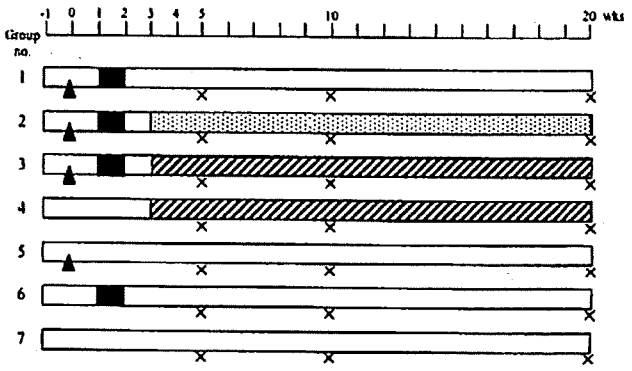


FIGURE 2 – Experimental protocol. ▲: AOM (10 mg/kg i.p.); ■: 2% DSS in drinking water; □: Basal diet and tap water; □: 1 ppm pitavastatin in diet; ▨: 10 ppm pitavastatin in diet; ×: Sacrifice.

*Immunohistochemistry*

Immunohistochemistry for proliferating cell nuclear antigen (PCNA)-positive nuclei, apoptotic nuclei, and nitrotyrosine-positive cells was performed on 4-μm-thick paraffin-embedded sections, from the colons of mice in each group by the labeled strepravidin biotin method, using a LSAB KIT (DAKO Japan, Kyoto, Japan), with microwave accentuation. The paraffin-embedded sections were heated for 30 min at 65°C, deparaffinized in xylene and rehydrate through grade ethanols at room temperature. A 0.05 M Tris HCl buffer (pH 7.6) was used to prepare solutions and for washes between various steps. Incubations were performed in a humidified chamber. For the determination of PCNA-incorporated nuclei, the PCNA-immunohistochemistry was performed. Apoptotic index was also evaluated by immunohistochemistry for single-stranded DNA (ssDNA). Sections were treated for 40 min at room temperature, with 2% BSA, and incubated overnight at 4°C with primary antibodies, anti-PCNA mouse monoclonal antibody (PC10, 1:50 dilution, DAKO Japan), anti-ssDNA rabbit polyclonal antibody (1:300 dilution, DAKO Japan) and anti-nitrotyrosine rabbit polyclonal antibody (1:500 dilution, Update Biotechnology, Lake Placid, NY). To reduce the nonspecific staining of mouse tissue by a mouse antibody (anti-PCNA), a Mouse On Mouse IgG blocking reagent (Vector Laboratories, Burlingame, CA) was applied for 1 hr. House-radish peroxidase activity was visualized by treatment with $H_2O_2$ and 3,3′-diaminobenzidine for 5 min. At the last step, the sections were weakly counterstained with Mayer's hematoxylin (Merck, Tokyo, Japan). For each case, negative controls were performed on serial sections. On the control sections, incubation with the primary antibodies was omitted.

Intensity and localization of immunoreactivity against all primary antibodies used were assessed using a microscope (Olympus BX41, Olympus Optical, Tokyo, Japan). The indices for PCNA and apoptosis were determined by counting the number of positive nuclei among at least 200 cells in 5 adenocarcinomas developed at Week 20 from each of Groups 1–3, and were indicated as percentages. The nitrotyrosine-positive cells were evaluated for their intensity of immunoreactivity on a 0 or 4+ scale. The overall intensity of the staining reaction was scored with 0 indicating no immunoreactivity and no positive cells, 1+ weak immunoreactivity and <10% of positive cells, 2+ mild immunoreactivity and 10–30% of positive cells, 3+ moderate immunoreactivity and 31–60% of positive cells and 4+ strong immunoreactivity and 61–100% of positive cells. This evaluation was done on the colonic mucosa with or without tumors from all the mice of each sacrifice time point (4 mice each from all groups at Week 5; 4 mice each from all groups at Week 10; and 9 mice each of Groups 1 and 3, 10 mice each of Groups 2 and 6, and 5 mice each of Groups 4, 5 and 7 at Week 20).

TABLE I – BODY, LIVER WEIGHT AND LENGTH OF LARGE BOWEL OF MICE AT WEEK 20

| Group no. | Treatment (no. of mice examined) | Body weight (g) | Liver weight (g) | Relative liver weight (g/100 g body weight) | Length of colon (cm) |
|---|---|---|---|---|---|
| 1 | AOM/2% DSS (9) | 44.02 ± 3.44[a] | 2.45 ± 0.34 | 5.56 ± 0.44 | 11.63 ± 0.41 |
| 2 | AOM/2% DSS/1 ppm pitavastatin (7) | 43.09 ± 6.79 | 2.21 ± 0.26 | 5.15 ± 0.28 | 11.66 ± 0.61 |
| 3 | AOM/2% DSS/10 ppm pitavastatin (9) | 38.40 ± 2.61 | 2.28 ± 0.32 | 5.94 ± 0.64[b] | 11.29 ± 0.86 |
| 4 | 10 ppm pitavastatin (5) | 42.47 ± 4.17 | 2.28 ± 0.23 | 5.39 ± 0.27 | 11.70 ± 1.54 |
| 5 | AOM (5) | 53.26 ± 6.63[c] | 2.50 ± 0.40 | 4.68 ± 0.38[d] | 12.18 ± 0.47 |
| 6 | 2% DSS (7) | 44.16 ± 5.12 | 2.45 ± 0.30 | 5.59 ± 0.76 | 11.13 ± 0.28 |
| 7 | None (4) | 42.84 ± 4.23 | 2.40 ± 0.32 | 5.58 ± 0.23 | 12.78 ± 0.17 |

[a]Mean ± SD.–[b]Significantly different from Group 2 by Tukey–Kramer multiple comparison post test ($p < 0.05$).–[c]Significantly different from Groups 1, 6, and 7 by Tukey–Kramer multiple comparison post test ($p < 0.05$).–[d]Significantly different from Group 1 by Tukey–Kramer multiple comparison post test ($p < 0.05$).

TABLE II – INCIDENCE OF COLONIC LESIONS AT WEEKS 5, 10 AND 20

| Group no. | Treatment (no. of mice examined at wk 5/wk 10/wk 20) | Mucosal ulcer | | | Dysplasia | | |
|---|---|---|---|---|---|---|---|
| | | Wk 5 | Wk 10 | Wk 20 | Wk 5 | Wk 10 | Wk 20 |
| 1 | AOM/2% DSS (4/4/9) | 4/4, 100% | 4/4, 100% | 6/9, 67% | 4/4, 100% | 4/4, 100% | 9/9, 100% |
| 2 | AOM/2% DSS/1 ppm pitavastatin (4/4/10) | 4/4, 100% | 3/4, 75% | 3/10, 30% | 4/4, 100% | 4/4, 100% | 8/10, 80% |
| 3 | AOM/2% DSS/10 ppm pitavastatin (4/4/9) | 2/4, 50% | 3/4, 75% | 0/9, 0% | 3/4, 75% | 4/4, 100% | 9/9, 100% |
| 4 | 10 ppm pitavastatin (4/4/5) | 0/4, 0% | 0/4, 0% | 0/5, 0% | 0/4, 0% | 0/4, 0% | 0/5, 0% |
| 5 | AOM (4/4/5) | 0/4, 0% | 1/4, 25% | 0/5, 0% | 1/4, 25% | 0/4, 0% | 0/5, 0% |
| 6 | 2% DSS (4/4/10) | 4/4, 100% | 4/4, 100% | 0/10, 0% | 1/4, 25% | 0/4, 0% | 0/10, 0% |
| 7 | None (4/4/5) | 0/4, 0% | 0/4, 0% | 0/5, 0% | 0/4, 0% | 0/4, 0% | 0/5, 0% |

Data were from histopathological analysis.

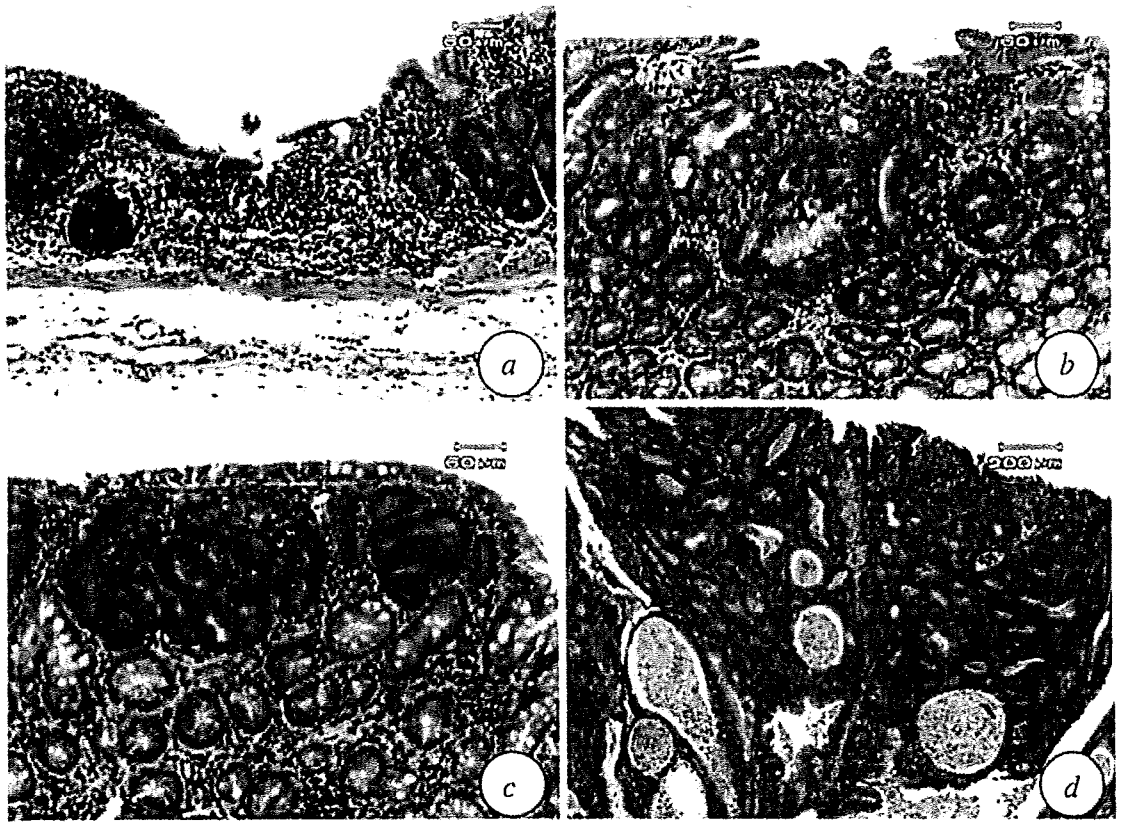


FIGURE 3 – Colonic lesions induced by AOM and 2% DSS. (*a*) A mucosal ulcer, (*b*) dysplastic crypts, (*c*) a tubular adenomas and (*d*) a tubular adenocarcinoma that developed in a mouse that received AOM and 2% DSS (Group 1). Bars inserted are (a) 60 μm, (b) 60 μm, (c) 60 μm and (d) 200 μm.

TABLE III – MULTIPLICITIES OF COLONIC LESIONS AT WEEKS 5, 10 AND 20

| Group no. | Treatment (no. of mice examined at wk 5/wk 10/wk 20) | Mucosal ulcer | | | Dysplasia | | |
|---|---|---|---|---|---|---|---|
| | | Wk 5 | Wk 10 | Wk 20 | Wk 5 | Wk 10 | Wk 20 |
| 1 | AOM/2% DSS (4/4/9) | 3.25 ± 1.71[a] | 2.75 ± 0.96 | 0.82 ± 0.98 | 4.50 ± 1.30 | 4.00 ± 1.41 | 3.18 ± 1.66 |
| 2 | AOM/2% DSS/1 ppm pitavastatin (4/4/10) | 2.00 ± 0.82 | 1.25 ± 0.96 | 1.50 ± 2.46 | 3.25 ± 0.50 | 3.30 ± 1.30 | 2.20 ± 1.99 |
| 3 | AOM/2% DSS/10 ppm pitavastatin (4/4/9) | 1.25 ± 1.50 | 0.75 ± 0.50[b] | 0 | 2.00 ± 1.83 | 3.75 ± 1.89 | 1.89 ± 0.93 |
| 4 | 10 ppm pitavastatin (4/4/5) | 0 | 0 | 0 | 0 | 0 | 0 |
| 5 | AOM (4/4/5) | 0 | 0.25 ± 0.50 | 0 | 0.25 ± 0.50 | 0 | 0 |
| 6 | 2% DSS (4/4/10) | 6.00 ± 2.16 | 3.75 ± 1.71 | 0 | 0.25 ± 0.50 | 0 | 0 |
| 7 | None (4/4/5) | 0 | 0 | 0 | 0 | 0 | 0 |

Data were from histopathological analysis.
[a]Mean ± SD.–[b]Significantly different from Group 1 by Tukey–Kramer multiple comparison post test ($p < 0.05$).

TABLE IV – INCIDENCE OF COLONIC TUMOR AT WEEKS 5, 10 AND 20

| Group no. | Treatment | Adenoma | | | Adenocarcinoma | | | Total | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Wk 5 | Wk 10 | Wk 20 | Wk 5 | Wk 10 | Wk 20 | Wk 5 | Wk 10 | Wk 20 |
| 1 | AOM/2% DSS (4/4/9) | 4/4, 100% | 3/4, 75% | 9/9, 100% | 4/4, 100% | 3/4, 75% | 9/9, 100% | 4/4, 100% | 4/4, 100% | 9/9, 100% |
| 2 | AOM/2% DSS/1 ppm pitavastatin (4/4/10) | 4/4, 100% | 4/4, 100% | 9/10, 90% | 3/4, 75% | 3/4, 75% | 9/10, 90% | 4/4, 100% | 4/4, 100% | 9/10, 90% |
| 3 | AOM/2% DSS/10 ppm pitavastatin (4/4/9) | 2/4, 50% | 4/4, 100% | 7/9, 78% | 2/4, 50% | 4/4, 100% | 7/9, 78% | 2/4, 50% | 4/4, 100% | 8/9, 89% |
| 4 | 10 ppm pitavastatin (4/4/5) | 0/4, 0% | 0/4, 0% | 0/5, 0% | 0/4, 0% | 0/4, 0% | 0/5, 0% | 0/4, 0% | 0/4, 0% | 0/5, 0% |
| 5 | AOM (4/4/5) | 0/4, 0% | 0/4, 0% | 0/5, 0% | 0/4, 0% | 0/4, 0% | 0/5, 0% | 0/4, 0% | 0/4, 0% | 0/5, 0% |
| 6 | 2% DSS (4/4/10) | 0/4, 0% | 0/4, 0% | 0/10, 0% | 0/4, 0% | 0/4, 0% | 0/10, 0% | 0/4, 0% | 0/4, 0% | 0/10, 0% |
| 7 | None (4/4/5) | 0/4, 0% | 0/4, 0% | 0/5, 0% | 0/4, 0% | 0/4, 0% | 0/5, 0% | 0/4, 0% | 0/4, 0% | 0/5, 0% |

Data were from histopathological analysis.

*Statistical analysis*

The incidences among the groups were compared using $\chi^2$ test or Fisher's extract probability test (GraphPad Instat version 3.05, GraphPad Software, San Diego, USA). Other measurements expressing mean ± SD were statistically analyzed using Tukey–Kramer multiple comparison post test (GraphPad Instat version 3.05, GraphPad Software). Differences were considered statistically significant at $p < 0.05$.

## Results

*General observation*

The animals that received DSS in their drinking water (Groups 1, 2, 3 and 6) had bloody stool between Weeks 1–3. Also, some mice treated with AOM/DSS with or without pitavastatin (Groups 1, 2 and 3) had bloody stool, and tumors developed in their colon. However, other groups including Group 4 (the pitavastatin alone group) did not have such symptom. Body weights, liver weights, and relative liver weights in all groups at Week 20 are shown in Table I. With regard to the mean body weights, Group 5 (the AOM alone group, 53.3 ± 6.6 g) significantly increased when compared with all other groups. However, the mean liver weight did not significantly differ among the groups, whereas the mean relative liver weight (g liver weight/100 g body weight) of Group 3 (the AOM/DSS/10 ppm pitavastatin group, 5.94 ± 0.64) was significantly greater than that of Group 2 (the AOM/DSS/1 ppm pitavastatin group, 5.15 ± 0.28, $p < 0.05$), and the value of Group 5 (4.68 ± 0.38) was significantly lower than that of Groups 1 (the AOM/DSS group, 5.56 ± 0.44, $p < 0.05$) and 3 (5.94 ± 0.64, $p < 0.01$). As shown in Table I, the mean length of the colon did not significantly differ among the groups.

*Incidence and multiplicity of colonic mucosal ulcer and dysplasia*

Table II summarizes the incidence of colonic mucosal ulcer (Fig. 3*a*) and colonic dysplasia (Fig. 3*b*) at each time point. The incidence of mucosal ulcer gradually decreased as administration of pitavastatins doses increased at each time-point. On the other hand, the incidence of dysplasia were unaffected. As given in Table III, the multiplicity of mucosal ulcer in Groups 1, 2, 3 and 6 was the highest at Week 5, and then they gradually decreased. At Weeks 5 and 10, the value was decreased by administration of pitavastatin dose-dependently. The multiplicity of mucosal ulcer of Group 3 ($p < 0.05$) was significantly decreased when compared with Group 1. At Week 20, mucosal ulcer was not found in mice of Group 3. Dysplastic crypts were also present in mice given AOM and DSS with or without pitavastatin treatment at Week 5. Colonic dysplasia tended to decrease during the experiment, as did mucosal ulcer. The multiplicities of dysplasia in the mice of Groups 2 and 3 were lower than that of Group 1, but the differences among the groups did not reach statistical significance.

*Incidence and multiplicity of large bowel neoplasms*

Table IV shows the incidence of colonic tumor at each time-point. It was observed that adenoma (Fig. 3*c*) and adenocarcinoma (Fig. 3*d*) located in the middle and distal colon at each time point. However, treatment with pitavastatin unaffected the incidence of colonic tumor at Weeks 10 and 20. The multiplicities of colonic neoplasms at Weeks 5, 10 and 20 are given in Table V. Colonic adenoma and adenocarcinoma were observed even at Week 5. The multiplicities of adenoma in Groups 2 and 3 were smaller than that of Group 1 at weeks 5 and 20, but the differences were not statistically significant among the groups. As for the

TABLE V – MULTIPLICITIES OF COLONIC TUMOR AT WEEKS 5, 10 AND 20

| Group no. | Treatment (no. of mice examined at wk 5/wk 10/wk 20) | Adenoma | | | Adenocarcinoma | | | Total | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Wk 5 | Wk 10 | Wk 20 | Wk 5 | Wk 10 | Wk 20 | Wk 5 | Wk 10 | Wk 20 |
| 1 | AOM/2% DSS (4/4/9) | 4.00 ± 1.15[a] | 2.25 ± 1.71 | 3.82 ± 1.78 | 3.00 ± 1.63 | 5.30 ± 1.30 | 5.27 ± 3.13 | 7.00 ± 1.15 | 7.50 ± 2.38 | 9.09 ± 3.86 |
| 2 | AOM/2% DSS/1 ppm pitavastatin (4/4/10) | 2.00 ± 0.82 | 2.25 ± 1.89 | 2.70 ± 1.42 | 2.30 ± 1.70 | 1.50 ± 1.29 | 1.50 ± 0.97[b] | 4.25 ± 2.06 | 3.75 ± 2.75 | 4.20 ± 2.10[c] |
| 3 | AOM/2% DSS/10 ppm pitavastatin (4/4/9) | 1.25 ± 1.50 | 3.75 ± 0.96 | 3.00 ± 1.87 | 1.50 ± 1.90 | 1.50 ± 1.00 | 2.00 ± 1.00[c] | 2.75 ± 3.20 | 5.25 ± 1.89 | 5.00 ± 2.50[d] |
| 4 | 10 ppm pitavastatin (4/4/5) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 5 | AOM (4/4/5) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 6 | 2% DSS (4/4/10) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 7 | None (4/4/5) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

All data were from histopathological analysis.
[a]Mean ± SD.—[b,c,d]Significantly different from Group 1 by Tukey–Kramer multiple comparison post test ([b]$p < 0.001$, [c]$p < 0.01$, and [d]$p < 0.05$).

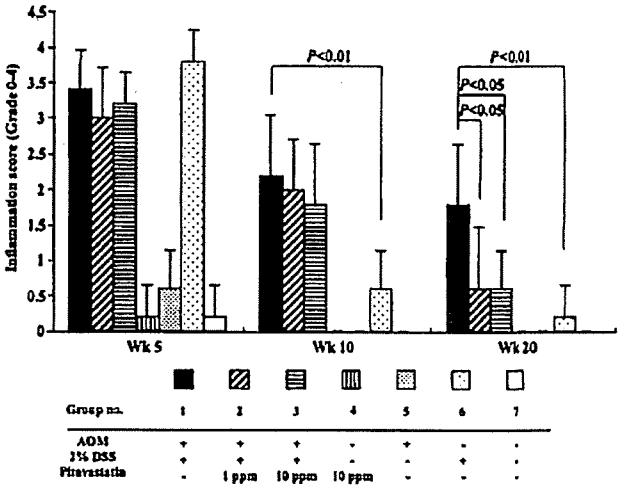


FIGURE 4 – Inflammatory scores in the large bowel of mice in all groups.

multiplicity of adenocarcinoma, the values of Groups 2 and 3 were low without statistical significance when compared to Group 1 at Weeks 5 and 10. However, the values of Groups 2 ($p < 0.001$) and 3 ($p < 0.01$) at Week 20 were significantly smaller than that of Group 1, although the inhibition was not dose-dependent.

*Inflammation score in the large bowel*

Figure 4 illustrates data on colonic inflammation scores at Weeks 5, 10 and 20. The inflammation scores of Groups 1, 2, 3 and 6 were the greatest at Week 5, and they gradually lowered with time. Colonic inflammation in the mice of Groups 4, 5 and 7, which were not given 2% DSS, were not observed at Weeks 10 and 20, while they had slight colitis at Week 5. At Weeks 5 and 10, the scores in Groups 2 and 3 that were given pitavastatin-containing diets were smaller than that of Group 1, but the differences did not reach the statistical significance. However, their scores were significantly lower than Group 1 at Week 20 (*vs.* Group 2, $p < 0.05$; Group 3, $p < 0.05$; and Group 6, $p < 0.01$).

*Immunohistochemical scores for PCNA-, ssDNA- and nitrotyrosine-positive cells in the colonic adenocarcinomas*

Scoring data on PCNA- (Fig. 5*a*) and ssDNA- (Fig. 5*b*) in adenocarcinoma cells and nitrotyrosine-positivity (Fig. 5*c*) in colonic mucosa with or without tumors are illustrated in Figure 6. As shown in Figure 6*a*, the mean PCNA-labeling indices of colonic adenocarcinomas developed in Groups 2 ($p < 0.001$) and 3 ($p < 0.001$) were significantly lower than that of Group 1. The mean apoptosis indices of Groups 2 ($p < 0.05$) and 3 ($p < 0.001$), which were measured by ssDNA immunohistochemistry, were significantly greater than that of Group 1, as shown in Figure 6*b*. Immunoreactivity of nitrotyrosine was noted in the adenocarcinoma cells (Fig. 5*c*). The reaction was also observed in the cryptal cells with or without disruption, infiltrated mononuclear inflammatory cells and endothelial cells of the small vessels in the mucosa and submucosa (Fig. 5*c*). The positive reaction was not detected in the colon of mice in Groups 4, 5 and 7. As illustrated in Figure 7, the scores of nitrotyrosine-positivity in Groups 1, 2, 3 and 6 were the greatest at Week 5, and decreased with time. At Week 5, the scores of Groups 2 ($p < 0.001$), 3 ($p < 0.001$) and 6 ($p < 0.05$) were significantly lower than that of Group 1. At Week 10, the scores of Groups 2 ($p < 0.01$), 3 ($p < 0.001$) and 6 ($p < 0.001$) were significantly lower than that of Group 1. Also, the scores of Groups 3 ($p < 0.05$) and 6 ($p < 0.01$) were significantly lower than that of Group 1 at Week 20.

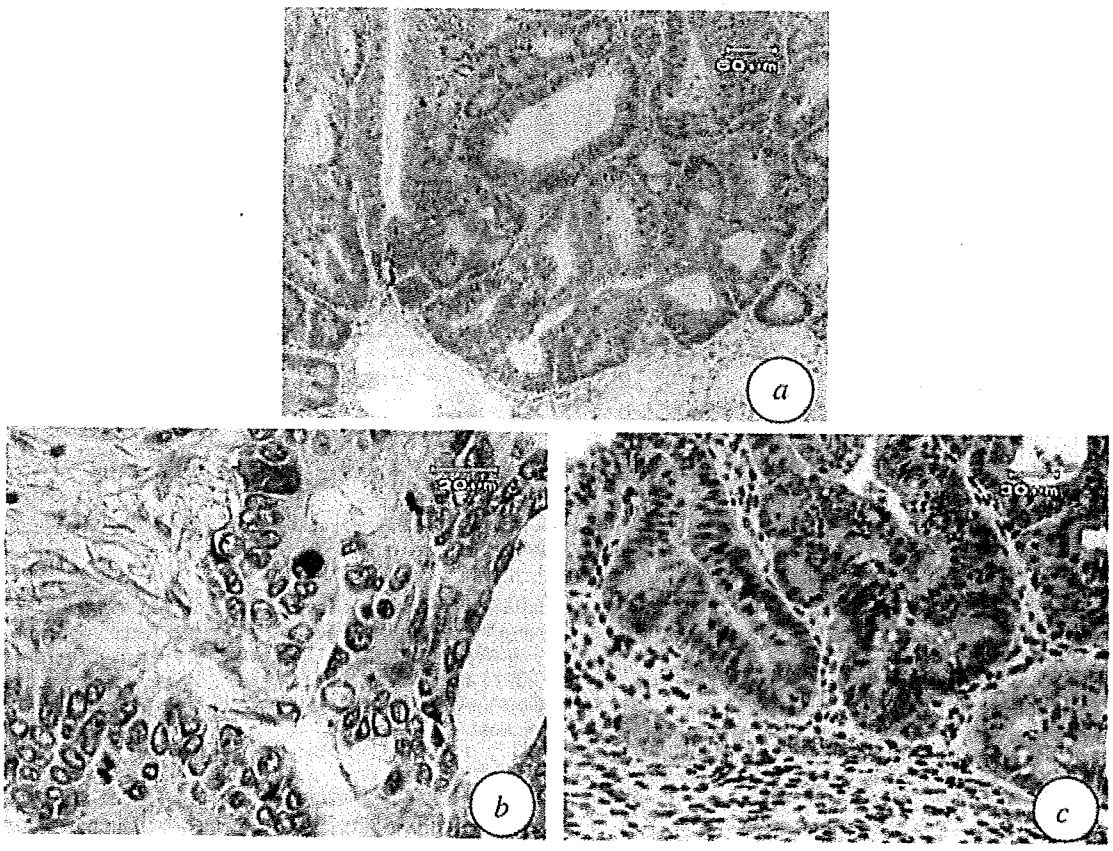


FIGURE 5 – Immunohistochemistry of (*a*) PCNA-labeled nuclei, (*b*) ssDNA-positive nuclei and (*c*) nitrotyrosine-positive cells in adenocarcinomas developed in the colon of a mouse from Group 1. Bars inserted are (a) 60 μm, (b) 20 μm and (c) 30 μm.

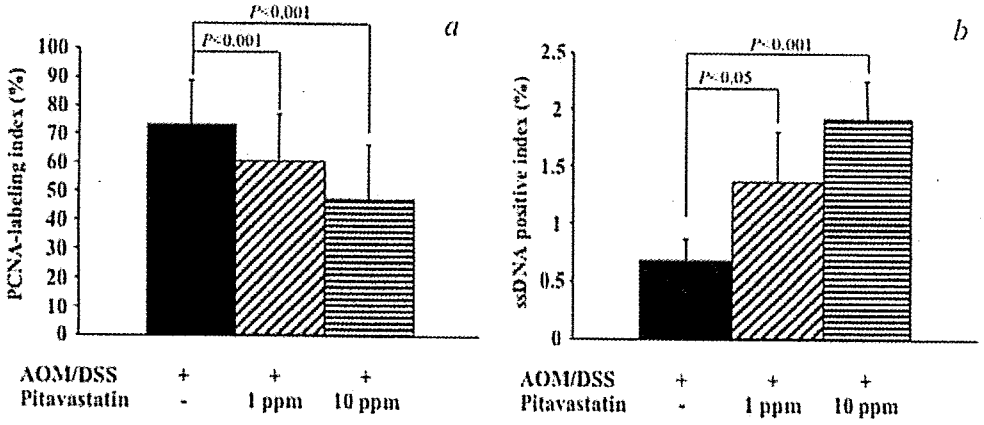


FIGURE 6 – Indices of (*a*) PCNA-labeled nuclei and (*b*) ssDNA-positive nuclei in 5 colonic adenocarcinomas each from Groups 1–3 at Week 20.

*Serum levels of TG and total cholesterol*

Table VI summarizes data on serum levels of TG and total cholesterol at each time point. The serum TG level of Group 1 (AOM/DSS group) was the greatest among the group at 3 time points. At Week 20, but not at Weeks 5 and 10, the values of Groups 2 ($p < 0.001$) and 3 ($p < 0.001$) were significantly lower than that of Group 1. Similarly, the serum level of total cholesterol of Group 3 ($p < 0.05$) was significantly smaller than that of Group 1, as listed in Table VII.

## Discussion

In the current study, we first demonstrated cancer chemopreventive effects of pitavastatin on colitis-related mouse colon carcinogenesis induced by AOM/DSS. Suppressing effects of colitis-related colon carcinogenesis by pitavastatin may be due to reduction of cell proliferation, induction of apoptosis, inhibition of inflammation and suppression of oxidative/nitrosative stress in the colonic malignancy. In the current study, treatment with pitavastatin alone (Group 4) did not affect colonic morphology, including

induction of ulcer and neoplasms. This is important, since a recent case report described the development of UC in a patient who took simvastatin and was fatal.[34]

In the current study, we observed that dietary pitavastatin inhibits the multiplicity, but not the incidence, of colonic adenocarcinomas induced by AOM/DSS. This may be related to weak chemopreventive effects of a low dose of pitavastatin. Also, there was no dose-response of the inhibition, although data on the indices of PCNA and ssDNA may suggest that pitavastatin affects dose-dependently proliferation and apoptosis in adenocarcinoma cells. Since only 2 doses (1 and 10 ppm in diet) of pitavastatin were used for assessing chemopreventive ability of the drug against AOM/DSS-induced mouse colon carcinogenesis in this study, additional doses (>10 ppm in diet) must be investigated to determine the dose-dependent efficacy of pitavastatin in suppressing AOM/DSS-induced colon carcinogenesis. As for colonic adenoma, the incidence did not significantly alter at 3 time points (Weeks 5, 10 and 20). The multiplicity of Group 2 was increased with time, but the increase was insignificant. The findings may suggest that a high dose (10 ppm) of pitavastatin is able to inhibit progress from adenoma to adenocarcinoma.

While statins are primarily known as drugs for the treatment of hypercholesterolemia because of their potency of reduction in LDL-cholesterol level by competitively inhibiting HMG-CoA reductase that is a rate-limiting enzyme in the synthesis of mevalonate, they have pleiotropic distinct effects on process such as angiogenesis[35] and inflammation.[36,37] Thus, statins affect a number of novel molecular targets and complex signaling pathways. Certain statins (simvastatin and rosuvastatin) are able to exert anti-inflammatory action in DSS-induced acute or chronic murine colitis model.[38,39] A lipophilic statin pitavastain also possesses multiple biological function[25] and anti-inflammatory action.[29,30] Pitavastatin is recently reported to down-regulate chemokines[40] that are involved in IBD pathogenesis.[41] Also, a low dose of pitavastatin can affect PI3K-AKT pathway,[42] which plays a critical role in the balance between cell survival and apoptosis, the inflammatory response by activating chemokine receptors and promoting inflammatory cell migration and the human cancer development,[43–45] including colon cancer.[46] In the current study, the treatment with pitavastatin in diet significantly lowered colonic inflammation induced by DSS, as revealed by histopathology (number of mucosal ulcer and inflammation scores). As observed in the colonic mucosa of UC patients, where colonic mucosal damage is associated with increased production of nitric oxide (NO) through the inducible nitric oxide synthase (iNOS) pathway,[47] the numbers of cryptal, infiltrated inflammatory, endothelial and tumor cells positive for nitrotyrosine, being a good biomarker for nitrosative stress,[48] were increased in the inflamed colon induced by DSS in this study. Pitavastatin treatment significantly lowered the nitrotyrosine-positive immunohistochemical score in conjunction with reduction in the number of mucosal ulcer and inflammatory score. iNOS is reported to be over-expressed in colonic tumors of humans[49] and chemically induced colonic tumors.[50] Although there are no reports that pitavastatin affects iNOs expression in inflamed tissues and neoplasms, our findings may suggest the possible effects of pitavastatin on iNOS expression. Activated nuclear factor-kappa B (NF-κB), which is a key player in inflammatory processes in the tissues,[51,52] is observed in different cancer cell lines and primary malignant tissue samples.[53] Recently, Lee *et al.*[38] demonstrated that simvastatin inhibits proinflammatory gene expression by blocking NF-κB signaling in intestinal epithelial cells, and attenuates DSS-induced acute murine colitis. Wang *et al.*[54] have also found that pitavastatin inhibits NF-κB activation and decreases IL-6 production induced by tumor necrosis factor-α in human hepatocellular carcinoma cells. NF-κB activation also plays an important role in enhancing IL-6 and IL-8 in human colon cancer cells.[55] Although we did not examine mRNA expression of NF-κB and cytokines in this study, it is possible that paitavastatin affects the expression in the inflamed mouse colon. The anti-inflammatory and antioxidative/nitrosative potential of pitavastatin is possibly related to prenylation of certain proteins that are involved in inflammatory processes,[56,57] but not its effect on HMG-CoA enzyme, as is the case of other statins.[17,58] The findings reported by others and those described here, thus, may suggest the potential use of statins, including pitavastatin as an anti-inflammatory drug for the treatment of IBD.

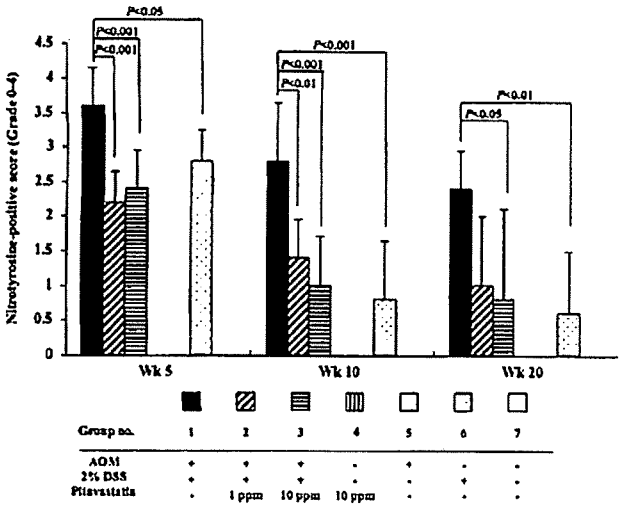


FIGURE 7 – Nitrotyrosine-positive indices in the colonic mucosa with or without tumors from all the mice of each sacrifice time point (4 mice each from all groups at Week 5; 4 mice each from all groups at Week 10; and 9 mice each of Groups 1 and 3, 10 mice each of Groups 2 and 6, and 5 mice each of Groups 4, 5 and 7 at Week 20).

Other interesting findings in the current study are that administration of pitavastatin induced apoptosis in the colonic epithelial malignancies. There are no reports describing apoptosis-inducing effects of pitavastatin on tumor cells, although certain statins possess proapoptotic properties in a variety of tumor cell lines.[59–62]

TABLE VI – SERUM TRIGLYCERIDE (MG/DL) AT WEEKS 5, 10 AND 20

| Group no. | Treatment | Wk 5 | Wk 10 | Wk 20 |
|---|---|---|---|---|
| 1 | AOM/2% DSS | 134.8 ± 63.5[a] (5) | 174.6 ± 96.7 (5) | 159.0 ± 59.7 (9) |
| 2 | AOM/2% DSS/1 ppm pitavastatin | 79.4 ± 27.5 (5) | 117.4 ± 21.7 (5) | 64.4 ± 16.8[b] (7) |
| 3 | AOM/2% DSS/10 ppm pitavastatin | 67.2 ± 26.8 (5) | 84.2 ± 28.0 (5) | 61.0 ± 27.5[b] (7) |
| 4 | 10 ppm pitavastatin | 77.8 ± 36.8 (5) | 67.2 ± 13.3 (5) | 59.0 ± 23.4 (5) |
| 5 | AOM | 126.0 ± 51.2 (5) | 92.0 ± 35.9 (5) | 94.8 ± 34.0 (5) |
| 6 | 2% DSS | 70.4 ± 33.4 (5) | 105.2 ± 24.8 (5) | 79.3 ± 37.9 (7) |
| 7 | None | 105.2 ± 38.1 (5) | 54.0 ± 15.3 (5) | 54.5 ± 16.0 (4) |

Numbers of parentheses are numbers of mice examined.
[a]Mean ± SD.–[b]Significantly different from Group 1 by Tukey–Kramer multiple comparison post test ($p < 0.001$).

TABLE VII – SERUM TOTAL CHOLESTEROL (MG/DL) AT WEEKS 5, 10 AND 20

| Group no. | Treatment | Wk 5 | Wk 10 | Wk 20 |
|---|---|---|---|---|
| 1 | AOM/2% DSS | 137.2 ± 10.0[a] (5) | 137.4 ± 22.7 (5) | 152.8 ± 43.7 (9) |
| 2 | AOM/2% DSS/1 ppm pitavastatin | 127.6 ± 14.8 (5) | 119.1 ± 20.9 (5) | 114.9 ± 18.2 (7) |
| 3 | AOM/2% DSS/10 ppm pitavastatin | 156.4 ± 26.2 (5) | 105.2 ± 10.5 (5) | 105.1 ± 23.5[b] (7) |
| 4 | 10 ppm pitavastatin | 117.2 ± 19.1 (5) | 109.0 ± 10.7 (5) | 106.6 ± 7.6 (5) |
| 5 | AOM | 134.8 ± 20.6 (5) | 146.4 ± 29.2 (5) | 161.4 ± 33.3 (5) |
| 6 | 2% DSS | 151.2 ± 28.2 (5) | 137.6 ± 35.4 (5) | 119.1 ± 20.3 (7) |
| 7 | None | 151.8 ± 14.6 (5) | 140.6 ± 18.4 (5) | 137.5 ± 25.1 (4) |

Numbers of parentheses are numbers of mice examined.
[a]Mean ± SD.–[b]Significantly different from Group 1 by Tukey–Kramer multiple comparison post test ($p < 0.05$).

Lipophilic statins are reported to induce apoptosis in malignant cells. For example, Agarwal *et al.*[59] reported that lovastatin induces apoptosis with differing sensitivity in a variety of colon cancer cell lines (SW480, HCT 116, LoVo and HT29). They also found that lovastatin treatment results in decreased expression of the antiapoptotic protein Bcl-2 and increased the expression of the proapoptotic protein Bax. There are some reports describing the comparison of apoptosis inducing ability between lipophilic and hydrophilic statins in tumor[60] and nontumor cells.[63,64] These reports suggested that lipophilic statins are more effective for inducing apoptosis when compared to hydrophilic statins. As to antiproliferative action of statins, the effect of lovastatin on prostate cancer cells is stronger than that of a hydrophilic statin, pravastatin.[65] Thus, the lipophilic property of pitavastatin may be related to the apoptosis induction and inhibition of proliferation in adenocarcinomas observed in this study.

Statins, including pitavastatin, are drugs that primarily affect LDL-cholesterol levels in plasma through the induction of the hepatic LDL receptor.[66] In this experiment, pitavastatin treatment effectively lowered serum total cholesterol level at Week 20. In addition, administration of pitavastatin significantly decreased serum TG level that was 3-fold increased by AOM/DSS exposure at Week 20. Hypertriglyceridemia is a risk for human CRC development.[67,68] Also, hyperlipidemia is a relatively frequent complication in patients with familial adenomatous polyposis patients.[69] In this context, a recent report[70] that lipoprotein lipase gene polymorphism influences lipid metabolism in UC patients and age of onset of UC is of interest.

A growing body of literature has emerged on the prevention of CRC in patients with long-standing CD and UC.[71,72] However, the data are not definitive and consist almost exclusively of retrospective case–control and cohort studies rather than the more rigorous prospective multiple randomized controlled trials.[31] Although the data on statins use are still too limited to endorse its use for the prevention of colitis-related CRC, further studies with statins need to be performed to develop an optimal strategy for the reduction of cancer risk in IBD patients. While most statins are metabolized in part by one or more hepatic cytochrome P450 enzymes (mainly CYP3A4), leading to an increased potential for drug interactions and problems with certain foods, such as grapefruit juice, pitavastatin appears to be metabolized by a substrate of CYP2C9.[25] This property may prove beneficial for the long-term use of the drug in clinic.

In conclusion, our current findings that a lipophilic statin pitavastatin was effective for inhibiting colitis-related mouse colon carcinogenesis through modulating the cell proliferation, mucosal inflammation and oxidative/nitrosative stress in the target tissue suggest possible application of pitavastatin in suppressing colon carcinogenesis in the inflamed colon of patients with IBD. Further studies on detailed mechanisms of the action involved are underway in our laboratory using microarray and proteomics techniques.

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# Tumor-initiating potency of a novel heterocyclic amine, aminophenylnorharman in mouse colonic carcinogenesis model

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**A novel heterocyclic amine, 9-(4′-aminophenyl)-9*H*-pyrido[3,4-*b*]indole (aminophenylnorharman, APNH), which is formed from nonmutagenic 9*H*-pyrido[3,4-*b*]indole (norharman) and aniline, is mutagenic to bacteria and mammalian cells and potently carcinogenic in rats. APNH is detected in human urine samples, suggesting that humans are continuously exposed to APNH. In the present study, $^{32}$P-postlabelin analysis revealed that the levels of APNH-DNA adduct 24 hr after the treatment with APNH (1, 5 and 20 mg/kg body weight) in male ICR mice were increased in a dose-dependent manner in the colon and liver. Based on these findings, we determined the tumor-initiating potency of APNH in an inflammation-related and two-stage mouse colon carcinogenesis model. Male Crj: CD-1 (ICR) mice were given a single intragastric administration (1, 2, 5 or 10 mg/kg body weight) of APNH and subsequent 1-week oral exposure to dextran sodium sulfate (DSS, 2% in drinking water). Treatment with APNH and DSS resulted in numerous colon tumor development: the incidence and multiplicity of the tumors were the highest in the mice received 10 mg/kg body weight of APNH and followed by DSS. Development of colon tumors was dose-dependent of APNH. Seven of 9 (77.8%) colonic adenocarcinomas developed in mice treated with APNH (10 mg/kg body weight) and DSS had β-*catenin* gene mutations at codons 32 and 37, being predominantly transversion. These findings indicate that APNH has an initiating activity in inflamed mouse colon and the APNH-DNA adduct formation correlates with its tumorigenic potential.**


**Key words:** aminophenylnorharman; adenocarcinoma; β-catenin; colon; dextran sodium sulfate; DNA adduct; heterocyclic amines; initiation; mice

The development of most human cancer might be caused by carcinogenic agents in the diet and cigarette smoke.[1] Therefore, identification of mutagens and carcinogens in foods and cigarette smoke is very important for understanding the causal agents of human cancer. A series of mutagenic and carcinogenic heterocyclic amines (HCAs) have been identified in cooked foods and heating amino acids and proteins.[2] The β-carboline compound norharman (9*H*-pyrido[3,4-*b*]indole) is produced in the pyrolysis of tryptophan.[3] Norharman is present at much higher levels than 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) in human urine samples.[4] Although norharman is not mutagenic to *Salmonella* strains, it becomes mutagenic to *S. typhimurium* TA98 in the presence of S9 mix, when incubated with non-mutagenic aromatic amines, such as aniline.[5] A novel HCA, 9-(4′-aminophenyl)-9*H*-pyrido[3,4-*b*]indole (aminophenylnorharman, APNH) can be formed by this reaction, then converted to the *N*-hydroxyamino derivative which produces DNA adducts after esterification to induce mutations in *S. typhimurium* TA98 and YG1024.[6–8] APNH is able to induce sister chromatid exchanges and chromosome aberrations.[9] We have recently demonstrated that APNH forms DNA adducts at the C-8 position of guanine residues in the various tissues including liver and colon of F344 rats after administration of APNH.[10] Subsequently, it has been reported that the *gpt* mutant frequencies were elevated in the liver and colon of the *gpt* delta transgenic mouse treated with APNH.[11] Norharman is reported to be present at much higher levels than those of HCAs in cooked meat and fish and cigarette smoke condensate.[12] Aniline is also present in cigarette smoke condensate and certain vegetables.[13] In addition, APNH was detected in 24 hr urine samples collected from F344 rats that received norharman and aniline.[14] Moreover, we have detected APNH in human urine samples from both healthy volunteers and patients in the hospital,[15] suggesting thus that humans are continuously exposed to APNH.

To understand the effects of APNH on human health, it is important to elucidate its carcinogenicity in rodents. It has been demonstrated that dietary feeding with APNH induces preneoplastic hepatocellular lesions, glutathione *S*-transferase placental form-positive foci, in the liver of male F344 rats.[16] Moreover, it has been already reported that the feeding with APNH for 85 weeks resulted in the development of hepatocellular carcinomas (HCCs, 10–79% incidence), and the colonic adenocarcinomas with low incidences (3–13% incidence) in male and female F344 rats.[17] These findings suggest a weak tumor-initiating capability of APNH in the colon.

We recently developed a novel mouse model for inflammation-related colon carcinogenesis[18] and this can be used for detecting the HCAs with colonic carcinogenicity in mice within a short-term period.[19] Also, we have evaluated tumor-promoting activity of chemicals utilizing this inflammation-related and two-stage mouse model.[20]

In the present study, we examined the APNH-DNA adduct formation in the colon and liver of mice that received APNH using the $^{32}$P-postlabeling method. Additionally, we investigated tumor-initiating activity of APNH in mice by gavage with various doses of APNH in the inflamed colon induced by dextran sodium sulfate (DSS). The β-*catenin* gene mutations in colonic adenocarcinomas developed were also analyzed by the single strand conformation polymorphism (SSCP) method and direct sequencing.

## Material and methods

### *Animals, chemicals and diet*

Male Crlj: CD-1 (ICR) mice (Charles River Japan, Tokyo, Japan) aged 5 weeks were used. They were maintained at Kanazawa Medical University Animal Facility according to the Institutional Animal Care Guidelines. All animals were housed in plastic cages

*Abbreviations:* APNH, aminophenylnorharman (9-(4′-amino-3′-aminophenyl)-9*H*-pyrido[3,4-*b*]indole); DSS, dextran sodium sulfate; HCA, heterocyclic amine; H&E, hematoxylin and eosin; i.g., intragastric; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; PCR, polymerase chain reaction; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; RAL, relative adduct labeling; SSCP, single strand conformation polymorphism.

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(4 or 5 mice/cage) with free access to drinking water and a pelleted basal diet, CRF-1 (Oriental Yeast, Tokyo, Japan), under controlled conditions of humidity (50% ± 10%), light (12/12 hr light/dark cycle) and temperature (23°C ± 2°C). After 7-days-quarantine, they were randomized by body weight into experimental and control groups. APNH was purchased from the Nard Institute (Osaka, Japan) and its purity was confirmed to be > 99% by HPLC. DSS with a molecular weight of 36,000–50,000 (Cat No. 160110) was purchased from MP Biochemicals, LLC (Aurora, OH).

*Formation of APNH-DNA adducts*

To analyze APNH-DNA adduct formation, nine male ICR mice were provided. At 6 weeks of age, they were given a single intragastric intubation (i.g.) of APNH at doses of 1, 5 and 20 mg/kg body weight and sacrificed after 24 hr. The liver and colon were excised and store at −80°C until DNA extraction by standard procedure involving enzymatic digestion of protein and RNA followed by extraction with phenol and chloroform/isoamyl alcohol (24:1, v/v).

DNA obtained from the experiment was digested with micrococcal nuclease and phosphodiesterase II, and the digest was $^{32}$P-post-labeled under modified-standard and adduct-intensification conditions as reported previously.[10,21,22] Adducts were detected with a Bio-Image Analyzer (BAS 2000; Fuji Photo Film, Tokyo, Japan) after exposing the TLC sheets to Fuji imaging plates. Relative adduct labeling (RAL) was determined by the method of Randerath *et al.*,[23] and the values were calculated as averages of 3 assays.

*Experimental procedure for tumor-initiating activity*

A total of 53 male ICR mice were divided into 7 experimental and solvent control groups. Groups 1 through 4 were given a single i.g. of APNH at a dose of 1, 2, 5 or 10 mg/kg body weight. Starting one week after the APNH administration, animals in Groups 1 through 4 were given 2% (w/v) DSS in drinking water for 7 days, and then followed without any further treatment for 17 weeks. Groups 5 and 6 were given APNH (10 mg/kg body weight) alone and 2% DSS alone, respectively. Group 7 was untreated. All animals were sacrificed at week 20. The colon were flushed with saline, excised, measured their length (from ileocecal junction to the anal verge), cut open longitudinally along the main axis and then washed with saline. After careful macroscopic inspection on the colon, they were cut and fixed in 10% buffered formalin for at least 24 hr. Histological examination was performed on paraffin-embedded sections after hematoxylin and eosin (H&E) staining. Some tumors were stored in a deep-freezer at −80°C for analysis of β-*catenin* mutation. On the H&E-stained sections, histological alterations, such as mucosal ulceration, dysplasia and colonic neoplasms were examined. Colonic neoplasms were diagnosed according to the description by Ward.[24] A histopathological examination was also done in other organs. Mucosal inflammation with or without ulceration in the colon was analyzed on the H&E-stained sections. Colonic inflammation was graded according to the following morphological criteria described by Cooper *et al.*[25]: Grade 0, normal appearance; Grade 1, shortening and loss of the basal one-third of the actual crypts with mild inflammation in the mucosa; Grade 2, loss of the basal two-thirds of the crypts with moderate inflammation in the mucosa; Grade 3, loss of the entire crypts with severe inflammation in the mucosa and submucosa, but with retainment of the surface epithelium; and Grade 4, presence of mucosal ulcer with severe inflammation (neutrophil, lymphocyte and plasma cell infiltration) in the mucosa, submucosa, muscularis propria and/or subserosa. The scoring was made on the entire intestine with or without proliferative lesions and expressed as a mean average score/mouse.

*DNA extraction*

For analysis of β-*catenin* mutations, 9 colonic adenocarcinomas (frozen materials) developed in APNH (10 mg/kg body weight)/DSS-treated mice were used. DNA was extracted from frozen tissue using Wizard® Genomic DNA Purification Kit (Promega, Madison, WI).

*Polymerase chain reaction (PCR)-SSCP analysis and direct sequencing*

DNA from colonic adenocarcinomas was PCR-amplified with primers (5′-primer, GCTGACCTGATGGAGTTGGA; 3′-primer, GCTACTTGCTCTTGCGTGAA), which were designed to amplify exon 3 of the β-*catenin* gene containing the consensus sequence for glycogen synthase kinase-3β phosphorylation.[26] The length of the PCR product with these primers is 227 bp. The primers were synthesized with a 394 DNA/RNA synthesizer (Applied Biosystems, Foster City, CA) and purified with an OPC cartridge (Applied Biosystems). PCR for non-radioisotopic SSCP was performed in 50 µl of reaction mixture consisting of 0.5 µM of each primer, 1 × PCR buffer (Perkin Elmer, Applied Biosystems Division, Foster City, CA), 200 µM each dNTP, 2.5 U AmpliTaq Gold™ (Perkin Elmer) and 0.5–5 µl of template DNA. The mixture was heated at 94°C for 9 min and subjected to 40 or 35 cycles of denaturation (94°C, 1 min), annealing (55°C, 2 min) and extension (72°C, 3 min) using a using a Perkin Elmer-Cetus thermal cycler. The PCR products were purified and concentrated to 20 µl using Microcon 100 (Amicon, Beverley, MA). Ten volumes of 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol were added to 0.5 µl of purified PCR products, heated to 90°C for 3 min and applied to 10% polyacrylamide gels containing 5% glycerol. Electrophoresis was carried out at 300 V for 2 hr at 20°C and the gels were soaked in 10% trichloroacetic acid and in 50% methanol for 10 min each. DNA bands were detected by silver staining using 2D Silver Staining Solution II (Daiichi Chemical DNA, Tokyo, Japan). When mutated shifted band were observed in the gels, mutation analysis was performed by direct sequencing of PCR products using on ALF express™ DNA sequencer (Pharmacia Biotech).

*Statistical analysis*

All measurements were compared by one-way ANOVA with either Tukey's correction or Fisher's exact probability test (GraphPad Instat version 3.05, GraphPad Software, San Diego, CA), with $p < 0.05$ as the criterion of significance.

## Results

*DNA adduct formation by APNH*

Administration of APNH had no macroscopic lesions in any organs. APNH-DNA adduct formation was analyzed in colon and liver of male ICR mice treated with APNH at doses of 1, 5 and 20 mg/kg body weight for 24 hr. When the DNA samples from the colon of APNH-treated mice were analyzed by $^{32}$P-postlabeling analysis, 2 major adduct spots corresponding to dG-C8-APNH were observed, and this TLC pattern was similar to that of DNA samples obtained from various tissues of rat received 40 ppm of APNH for 4 weeks.[10] Also, the TLC pattern of liver DNA samples were the similar to those of colon DNA samples. As shown in Table I, the adduct levels of the colon and liver were increased in a dose-dependent manner. Furthermore, the RAL values for colon were higher than those for liver.

TABLE I – LEVELS OF APNH-DNA ADDUCTS IN THE LIVER AND COLON ON MICE GIVEN APNH

| Treatment (no of mice examined) | Adduct levels (adducts/$10^7$ nucleotides) | |
|---|---|---|
| | Liver | Colon |
| APNH 1 mg/kg (3) | 0.39 ± 0.13[1] | 5.9 ± 2.6 |
| APNH 5 mg/kg (3) | 3.82 ± 2.30 | 45.2 ± 10.3 |
| APNH 20 mg/kg (3) | 41.0 ± 15.3 | 141.9 ± 45.8 |

[1]Means ± SD.

TABLE II – BODY WEIGHTS, LIVER WEIGHTS, AND LENGTHS OF COLON IN EACH GROUP

| Group no. | Treatment (no of mice examined) | Body weight (g) | Liver weight (g) | Length of colon 7(cm) |
|---|---|---|---|---|
| 1 | APNH 1 mg/kg→2%DSS (10) | 44.9 ± 4.9[1] | 2.99 ± 0.46[2] | 14.6 ± 0.8 |
| 2 | APNH 2 mg/kg→2%DSS (9) | 42.8 ± 3.2 | 2.74 ± 0.32 | 14.3 ± 0.7 |
| 3 | APNH 5 mg/kg→2%DSS (8) | 43.0 ± 6.1 | 2.75 ± 0.57 | 13.9 ± 1.0 |
| 4 | APNH 10 mg/kg→2%DSS (9) | 42.1 ± 3.8 | 2.56 ± 0.47 | 12.3 ± 0.9[3] |
| 5 | APNH 10 mg/kg (5) | 42.8 ± 3.6 | 2.31 ± 0.47 | 12.3 ± 0.9[3] |
| 6 | 2%DSS (7) | 42.3 ± 2.7 | 2.19 ± 0.22 | 13.3 ± 1.2 |
| 7 | Untreated (5) | 41.6 ± 2.8 | 2.45 ± 0.17 | 15.1 ± 0.5 |

[1]Mean ± SD.–[2]Significantly different from group 6 by Tukey's multiple comparison post test ([2]$p < 0.01$).–[3]Significantly different from group 7 by Tukey's multiple comparison post test ([3]$p < 0.001$).

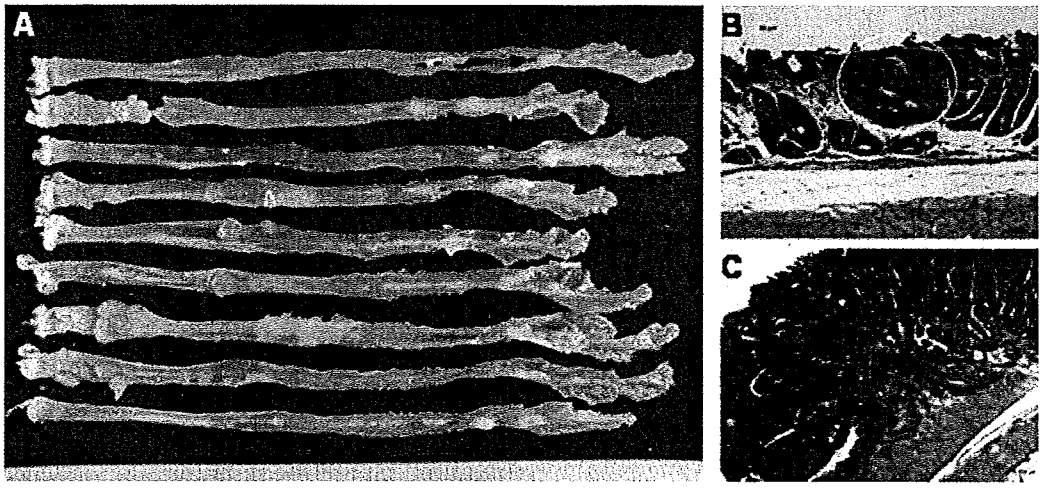


FIGURE 1 – Macroscopic view of the large bowel (*a*) and histopathology of the colonic lesions (*b*, *c*) of ICR mice treated with APNH 10 mg/kg body weight → 2% DSS. (*a*) A number of colonic tumors are seen in mice given APNH 10 mg/kg body weight → 2% DSS. (*b*) A tumor (white circle) is diagnosed as tubular adenoma. (*c*) A tumor is histologically well differentiated tubular adenocarcinomas. H&E stain, original magnification: (*b*), (*c*), × 100.

TABLE III – INCIDENCE OF VOLONIC NEOPLASMS IN MICE TREATED WITH APNH AND DSS

| Group no. | Treatment (no of mice examined) | No. of mice with colonic neoplasms | | |
|---|---|---|---|---|
| | | Total (multiplicity) | Adenoma (multiplicity) | Adenocarcinoma (multiplicity) |
| 1 | APNH 1 mg/kg→2%DSS (10) | 0% (0) | 0% (0) | 0% (0) |
| 2 | APNH 2 mg/kg→2%DSS (9) | 11% (0.11 ± 0.33) | 11% (0.11 ± 0.33) | 0% (0) |
| 3 | APNH 5 mg/kg→2%DSS (8) | 13% (0.38 ± 1.06) | 13% (0.13 ± 0.35) | 13% (0.25 ± 0.71) |
| 4 | APNH 10 mg/kg→2%DSS (9) | 56%* (1.89 ± 2.09[1,2]) | 33% (0.78 ± 1.39) | 56%* (1.11 ± 1.26[1,2]) |
| 5 | APNH 10 mg/kg (5) | 0 | 0 | 0 |
| 6 | 2%DSS (7) | 0 | 0 | 0 |
| 7 | Untreated (5) | 0 | 0 | 0 |

Numbers in parentheses are mean ± SD.
*Significantly different from group 6 by Fisher's exact probability test (*$p = 0.033$).
[1]Significantly different from group 1 by Tukey's multiple comparison post test ([1]$p < 0.01$).–[2]Significantly different from group 2 by Tukey's multiple comparison post test ([2]$p < 0.05$).

*General observation in the inflamed colon induced by DSS tumor-initiating activity*

Bloody stool was found during and soon after of DSS exposure (Day 12–21) in a few mice that received 2% DSS and their body weight gains were slightly decreased (data not shown). The body and liver weights and lengths of colon of mice in all groups at the end of the study (week 20) are listed in Table II. The mean body weights showed no significant differences among the groups. The means liver weights of Group 1 (APNH 1 mg/kg (2% DSS) was significantly larger than that of Group 6 (2% DSS alone, $p < 0.01$). The mean length of colon of mice in Group 4 (APNH 10 mg/kg → 2% DSS, $p < 0.001$) were significantly lower than that of Group 7 (untreated).

*Pathological findings in the inflamed colon induced by DSS tumor-initiating activity*

Macroscopically, nodular, polypoid or flat-type colonic tumors were observed in the middle and distal colon of mice in Groups 2 through 4 (Fig. 1*a*, Table III). Their histopathology was well- or moderately differentiated tubular adenocarcinoma (Fig. 1*b*) or tubular adenoma (Fig. 1*c*). There were no tumors in any organs other than colon in these groups. The mice in Group 1 (APNH 1 mg/kg → 2% DSS) had no tumors in the colon and other organs, including liver. The incidences of total colonic neoplasms and adenocarcinoma in Groups 4 (APNH 10 mg/kg → 2% DSS; $p < 0.05$) were significantly greater than Group 6 (2% DSS alone). Also, the multiplicity of colonic neoplasms and colonic adenocar-

TABLE IV – INCIDENCE OF COLON ULCERATION AND DYSPLASIA IN MICE TREATED WITH APNH AND DSS

| Group no. | Treatment (no of mice examined) | Inflammation score | Incidence of mucosal ulcer (multiplicity) | Incidence of colonic dysplasia | | |
|---|---|---|---|---|---|---|
| | | | | Total (multiplicity) | Low-grade (multiplicity) | High-grade (multiplicity) |
| 1 | APNH 1 mg/kg→2%DSS (10) | 2.00 ± 0.94 | 100% (3.30 ± 1.70) | 100%* (4.70 ± 1.57) | 100%* (3.90 ± 1.45) | 100%* (1.10 ± 4.70) |
| 2 | APNH 2 mg/kg→2%DSS (9) | 2.22 ± 0.97 | 100% (3.78 ± 1.92) | 100%* (6.22 ± 3.46) | 100%* (3.89 ± 1.45) | 100%* (2.33 ± 2.50) |
| 3 | APNH 5 mg/kg→2%DSS (8) | 2.13 ± 0.83 | 100% (3.88 ± 1.46) | 100%* (7.13 ± 2.80) | 100%* (3.75 ± 1.39) | 100%* (3.38 ± 2.13) |
| 4 | APNH 10 mg/kg→2%DSS (9) | 1.78 ± 0.44 | 100% (3.11 ± 1.05) | 100%* (9.11 ± 2.62[1]) | 100%* (4.44 ± 1.42) | 100%* (4.67 ± 2.12[1]) |
| 5 | APNH 10 mg/kg (5) | 0 | 0% (0) | 0% (0) | 0% (0) | 0% (0) |
| 6 | 2%DSS (7) | 1.86 ± 0.69 | 100% (3.00 ± 1.00) | 0% (0) | 0% (0) | 0% (0) |
| 7 | Untreated (5) | | 0% (0) | 0% (0) | 0% (0) | 0% (0) |

Numbers in parentheses are mean ± SD.
*Significantly different from group 6 by Fisher's exact probability test (*$p < 0.001$).
[1]Significantly different from group 1 by Tukey's multiple comparison post test ([1]$p < 0.05$).

cinoma in Group 4 (APNH 10 mg/kg → 2% DSS) were significantly greater than Groups 2 (APNH 2 mg/kg → 2% DSS; $p < 0.01$). The squared correlation coefficients for the multiplicities of total colonic neoplasms (adenoma plus adenocarcinoma), adenoma and adenocarcinoma obtained by the regression were 0.97 ($p < 0.01$), 0.95 ($p < 0.05$) and 0.98 ($p < 0.01$), respectively. These values suggested a dose-response tumor-initiating potency of APNH on development of colonic tumors. In mice of Groups 5–7, no neoplasms developed in any organs including colon.

As indicated in Table IV, colonic dysplasia also developed in Groups 1 through 4. Their multiplicities of the total dysplasia and high-grade dysplasia were significantly increased when the dose of APNH increased in Groups 1–4. The correlation coefficients for the multiplicities of total dysplasia and high-grade-dysplasia were 0.97 ($p < 0.01$) and 0.96 ($p < 0.01$), respectively. These values suggested a dose-response effect on the development of colonic dysplasia. There were no dysplatic lesions in mice of Groups 5 through 7. In addition, colonic mucosal ulceration was found in the distal colon of mice that received DSS (Table IV). Table IV summarizes data on colonic inflammation scores in the colon. No significant differences on the degrees of colonic mucosal inflammation were noted among the groups treated with DSS (Groups 1–4 and 6).

*Mutation in β-Catenin gene*

In the current experiment, mutations of exon 3 of the β-*catenin* gene were investigated in the colonic adenocarcinomas developed in mice of Group 4 (APNH 10 mg/kg (2% DSS) by the PCR-SSCP method and direct sequencing. Seven of 9 (77.8%) colonic adenocarcinomas developed had β-*catenin* gene mutations that were restricted to codons 32 and 37 (Table V). The mutations were GAT (Asp) to TAT (Tyr) and GAT (Asp) to AAT (Asn) at the codon 32 and TCT (Ser) to TGT (Cys) and TCT (Ser) to TAT (Tyr) at the codon 37. The majority of these mutations were transversions.

## Discussion

The results of the present study using an inflammation-related two-stage mouse colon carcinogenesis model indicate that one of the HCAs, APNH, has a strong tumor-initiation activity in the inflamed colon. This activity was dose-dependent and related with APNH-DNA adducts formation levels in the colon. The adenocarcinomas developed in mice initiated with APNH and promoted by DSS possessed β-*catenin* mutations. This is the first report demonstrating tumor-initiating ability of APNH in the colon. In this study, we also investigated the initiation activity of 20 mg/kg body weight of APNH. Although approximately half of the mice that received APNH (20 mg/kg body weight) followed by 2% DSS died due to hepatotoxicity of APNH 3–4 days after i.g. administration, the survivors had colonic tumors (50% incidence of adenocarcinoma with 0.73 ± 0.90 multiplicity and 33% incidence of adenomas with 0.55 ± 0.82 multiplicity).

DNA adduct formation by chemical carcinogens is important because the active dose of chemical carcinogen reaching its target site should exert their initiating events in chemical carcinogene-

TABLE V – MUTATIONS IN EXON 3 OF THE β-*CATENIN* GENE IN APNH/DSS-INDUCED MOUSE COLONIC ADENOCARCINOMAS

| Sample | β-catenin status | | Amino acid substitution |
|---|---|---|---|
| APNH /DSS-1 | Codon 37 | TCT→TGT | Ser→Cys |
| APNH /DSS-2 | Codon 37 | TCT→TGT | Ser→Cys |
| APNH /DSS-3 | Codon 32 | GAT→TAT | Asp→Tyr |
| APNH /DSS-4 | Codon 37 | TCT→TAT | Ser→Tyr |
| APNH /DSS-5 | | Wild type | |
| APNH /DSS-6 | Codon 32 | GAT→AAT | Asp→Asn |
| APNH /DSS-7 | Codon 32 | GAT→TAT | Asp→Tyr |
| APNH /DSS-8 | | Wild type | |
| APNH /DSS-9 | Codon 32 | GAT→TAT | Asp→Tyr |

sis.[27] The HCAs form tissue DNA adducts in humans and rodents.[28] MeIQx induces HCCs[29] and produces relatively high hepatic DNA adduct levels in rats.[30] In contrast, PhIP that is not hepatocarcinogenic, forms relatively low hepatic PhIP-DNA adduct levels in comparison with other tissues in rats.[31] APNH-DNA adducts were detected in various organs of rats given APNH, with levels higher in the liver and colon than in other organs.[10] In the current study, we observed the APNH-DNA adduct formation in the colon and liver, as found in rats,[10] and the levels in colon were greater than in liver. Furthermore, there was a clear dose-dependency in the APNH-DNA adduct formations in both organs. Thus, APNH-DNA adducts should be involved in the induction of colonic neoplasia in mice. APNH formed DNA adducts in the liver and induced HCC in F344 rats.[17] However, the mice received with APNH/DSS had no liver tumors in this study. These differences in the liver carcinogenicity of APNH may be due to the differences in the experimental protocol. As reported previously in rats,[10] the major APNH-DNA adduct was dG-C8-APNH in this study, it is suggested that dG-C8-APNH was formed in the target genes, such as β-*catenin*, and these adducts might cause the mutations. In fact, the mutations detected in the APNH-induced tumors were mainly at G:C base pairs.

HCAs, such as PhIP and MeIQx are able to induce multi-site tumors in rodents and monkeys.[32,33] When given high doses of PhIP for a long-term period, colonic tumors developed in rats.[34] Although dietary APNH at a dose of 20 or 40 ppm for 85 weeks induced colonic tumors in F344 rats, the incidence of colonic tumors was low, being 3 or 9% in males and 4 or 13% in females, respectively,[17] suggesting a weak tumor-initiating ability of APNH in the rat colon. In the current study, colonic neoplasms developed in the mice given APNH at all doses except for 1 mg/kg b.w., when followed by DSS. The tumor-initiating ability of APNH in this model was greater than that of PhIP and MeIQx when used the same experimental protocol.[19] These findings suggest the importance of inflammation in colon carcinogenesis. For further understanding the role of APNH in human colon cancer development, studies for APNH-DNA adduct levels in human colon samples and *in vivo* rat experiments with the protocol used in this study will be required.

Recent evidence demonstrates that the β-catenin signaling pathway is closely associated with the development of colon cancer.[35,36] The β-*catenin* gene is frequently mutated in human colon cancers without *APC* mutations[37] and carcinogens induced colorectal cancer in rodents.[26,38] In the present study, we detected β-*catenin* gene mutations of mouse colon adenocarcinomas induced by APNH/DSS, at codons 32 and 37, being transversions (G:C to T:A and G:C to C:G) and transition (G:C to A:T), and all gene mutations detected in APNH/DSS-induced colon adenocarcinomas in the present study involved G:C base pairs. In contrast, the location and mutation pattern were slightly different from a report documenting that the β-*catenin* gene mutations of rat colon tumors induced by APNH; G:C to A:T transition at codon 34 was predominant.[17] Masumura *et al.* has also reported that PhIP[39]- and APNH[11]-induced *gpt* mutations in the liver and colon of the *gpt* delta transgenic mice were dominated by G:C to T:A transversions.[40] The difference of APNH-induced mutation sites and patterns in rats and mice might reflect differences in DNA repair or translesion DNA synthesis between these 2 species. Moreover, the colonic inflammation observed in DSS mouse model could influence mutation patterns and hotspots induced by APNH. Recently, we detected the β-*catenin* mutations of mouse colon adenocarcinomas, induced by azoxymethane/DSS, at codons 32, 33 and 34, being G:C to A:T transitions.[19] In 1,2-dimethylhydrazine/DSS-induced mouse colon adenocarcinomas, mutations of the β-*catenin* gene were present in codons 32, 34, 37 and 41, being G:C to A:T transitions.[41] In contrast, mutations of the β-*catenin* were observed at codons 32 and 34, mainly being transversions (G:C to T:A and G:C to C:G) in PhIP/DSS-induced colon adenocarcinomas.[19] We could not detect β-*catenin* mutations in 2 adenocarcinomas in the current study, but there is a possibility that they have mutations in the genes other than β-*catenin*. Additionally, in human colorectal cancer, the β-*catenin* mutations were dominated by transitions.[37,42] Although reason for differences of β-*catenin* mutation patterns between colorectal cancers of human and mice remains unclear, the differences might be related to the low occurrence of β-*catenin* mutations in human colorectal cancers when compared with *APC* mutations[43] or the different pathways of tumorigenesis between sporadic cancer and inflammation-associated cancer.

In conclusion, the results in the current study indicate that a potential tumor-initiating activity of APNH in the inflamed colon. β-*Catenin* mutations in adenocarcinomas induced by APNH and DSS were different from those other colonic carcinogens including HCAs, possibly due to the adduct (dG-C8-APNH) formation. Norharman and aniline are abundantly present in our environment and continuous exposure to both compounds during daily life is conceivable. We recently detected APNH in human urine samples from both healthy volunteers and inpatients.[15] Although their APNH levels were independent of dietary intake or cigarette smoking,[15] APNH could be a novel type of endogenous mutagen/carcinogen, as shown in this study and a previous investigation.[17] The APNH level in human urine samples was much lower than the MeIQx level and PhIP level, but the level is comparable to that for 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1).[4,15] However, the carcinogenicity of APNH is thus considered to be more potent than that of PhIP and MeIQx in this animal model.[19] Our findings may provide scientific basis for further study on the involvement of APNH in human health.

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# A Tobacco-specific Carcinogen, NNK, Enhances AOM/DSS-induced Colon Carcinogenesis in Male A/J Mice

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**Abstract.** *To determine whether tobacco-derived carcinogens affect colon carcinogenesis, the effects of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) on colon carcinogenesis were examined using an azoxymethane (AOM)/dextran sulfate sodium (DSS) mouse model. NNK (10 μmol) was administered to male A/J mice by a single intraperitoneal (i.p.) injection and then AOM (10 mg/kg body weight, i.p.) was given 1 week after NNK administration. One week later, the mice received 1.5% (w/v) DSS in their drinking water for 7 days. All animals were sacrificed at week 22 to examine the pathological lesions in the colon and lung. The incidence (80%, $p<0.05$) and multiplicity (4.0±3.6, $p<0.05$) of colonic tumors of the NNK+AOM+DSS group were significantly higher than that of the AOM+DSS group (incidence, 40%; and multiplicity, 1.2±1.7). The differences in incidence and multiplicity of lung tumors were insignificant between these two groups. Our findings may suggest that smoking increases the risk of inflammation-related colon cancer development.*

Many studies generally show a positive association between tobacco use and mortality from colorectal cancer (CRC) (1, 2), despite ambiguities in that some studies have not shown a relation between tobacco and CRC development (3). Incidence of malignant neoplasm was higher by 1.5- to 3-fold in smokers than in non-smokers (4, 5). Tobacco use is known to cause many types of cancer in different organs, including lung. Smoking is a risk factor of cancer development in organs not in direct contact with smoke. Approximately 20% of the large bowel cancers in men would be attributable to smoking (6). Compared with never smokers, current smokers showed a 4-fold increase in risk of hyperplastic and adenomatous polyps in the colon (7). Furthermore, the risk of hyperplastic polyps and adenomas remained for up to 10 years after they stopped smoking (7). In spite of such findings, however, experimental evidence that tobacco smoking is involved in the pathogenesis of CRC is insufficient. 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is a tobacco-specific nitrosamine derived from nicotine, a major alkaloid in tobacco smoke (8, 9) and has been implicated as a major cause of tobacco-associated lung cancer (9, 10). Ye *et al.* (11) reported that NNK stimulated cell proliferation *via* 5-lipoxygenase (LOX) and cyclooxygenase (COX)-2 expressions in SW116 human colon cancer cells. However, it has not been elucidated whether NNK affects colon carcinogenesis *in vivo*.

Inflammatory bowel diseases (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), frequently progress to CRC (12, 13). Smoking is an important environmental factor in the pathogenesis of IBD (14, 15) and differently affects CD and UC: smoking increases the risk for CD and decreases that of UC (14, 15). In an animal model of colitis, chronic treatment with nicotine exerted biphagic effects on trinitrobenzene sulfonic acid-induced colitis (16): lower doses of nicotine were protective, but higher doses were deleterious. These findings may suggest that smoking can influence inflammation in the large bowel and, therefore, possibly affect CRC development in the inflamed colon.

In the current study, we investigated whether NNK affects inflammation-related colon carcinogenesis using our mouse model with azoxymethane (AOM) and dextran sulfate sodium (DSS) (17, 18). This animal model for colitis-related CRC is useful to investigate the modifying effects of xenobiotics on colitis-related colon carcinogenesis, since large bowel

*Abbreviations:* AD, adenoma; ADC, adenocarcinoma; AOM, azoxymethane; CD, Crohn's disease; COX, cyclooxygenase; CRC, colon cancer; DSS, dextran sulfate sodium; HP, hyperplasia; LOX, lipoxygenase; nAChR, nicotinic acetylcholine receptor; NFκB, nuclear factor-κB; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; ROS, reactive oxygen species; UC, ulcerative colitis.

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*Key Words:* Cigarette smoking, NNK, colitis-associated colon cancer.

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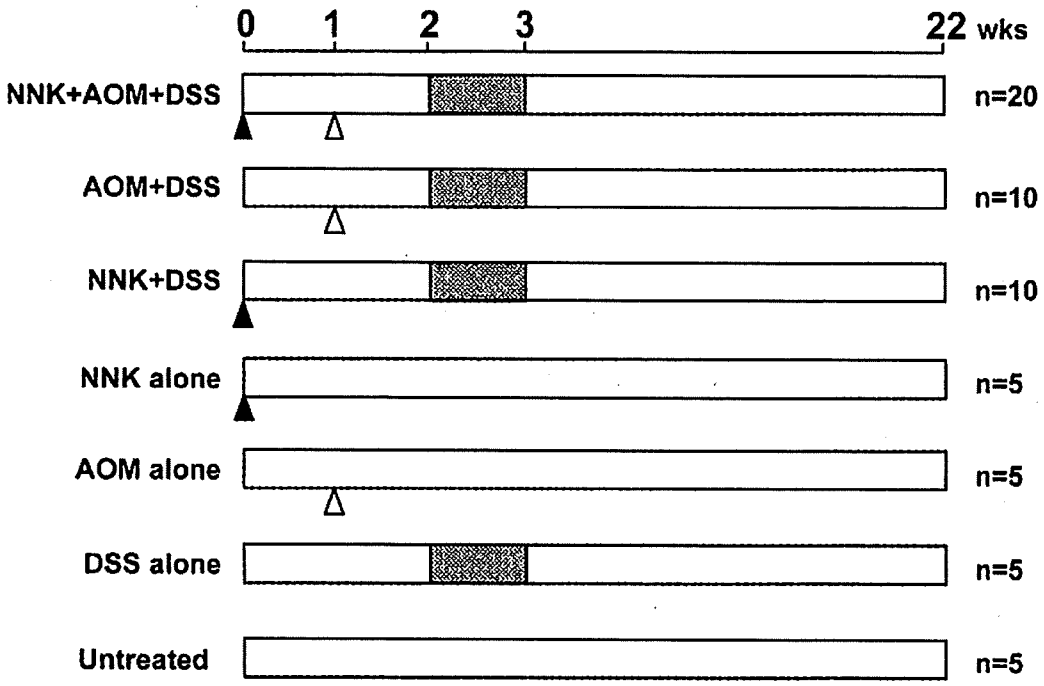


Figure 1. *Experimental protocol.* ▲ *NNK 10 μmol/mouse, i.p.;* △ *AOM 10 mg/kg bw, i.p.;* ▨ *1.5% DSS in drinking water.*

malignancies possessing biological alterations similar to those found in humans (18) develop within a short-term period.

## Materials and Methods

*Animals, chemicals and diets.* Five-week-old male A/J mice were purchased from Charles River Japan (Tokyo, Japan). They were maintained at the Kanazawa Medical University Animal Facility according to the Institutional Animal Care Guideline. All animals were housed in plastic cages (5 mice/cages) and given drinking water and a pelleted diet, CRF-1 (Oriental Yeast, Tokyo, Japan) *ad libitum*, under controlled conditions of humidity (50±10%), light (12/12 h light /dark cycle) and temperature (23±2°C). After arrival, they were quarantined for the first 7 days and then randomized by their body weights into experimental and control groups.

NNK was obtained from Toronto Research Chemical Inc. (Ontario, Canada). AOM was purchased from Sigma Chemical (St. Louis, MO, USA). DSS with a molecular weight of 36,000-50,000 (Cat. No. 160110) was purchased from MP Biomedicals, LLC (Aurora, OH, USA). DSS for induction of colitis was dissolved in water at a concentration of 1.5% (*w/v*).

*Experimental procedures.* A total of 60 male A/J mice were divided into 7 experimental and control groups (Figure 1). They were given a single intraperitoneal (*i.p.*) injection of NNK (10 μmol/mouse). After 7 days, animals were also treated with a single *i.p.* of AOM (10 mg/kg). Starting 7 days after AOM injection, 1.5% (*w/v*) DSS was given in the drinking water for 7 days. Experimental groups included: Group 1 (n=20) that received NNK, AOM and DSS; Group 2 (n=10) that were given AOM and DSS; Group 3 (n=10) was treated with NNK and DSS; Groups 4, 5 and 6 (n=5, each group) were given AOM alone, NNK alone, and DSS alone, respectively; and Group 7 (n=5) was an untreated control group. At week 22, all mice were sacrificed to histopathologically investigate proliferative lesions in the lung and large bowel. At sacrifice, the lung and large bowel were removed and macroscopically inspected for the presence of tumors. The lung (all lobes) was measured and fixed in 10% buffered formalin for a least 24 h. After measuring the length of large bowels (from the ileocecal junction to the anal verge), large bowels were cut open longitudinally along the main axis and gently washed with saline. They were then cut along the vertical axis and fixed in 10% buffered formalin for a least 24 h. Histopathological examination was performed on hematoxylin and eosin (H&E)-stained sections made from paraffin-embedded blocks. Colonic and lung tumors were diagnosed, according to Ward's (19) and Nikitin *et al's* descriptions (20), respectively.

*Statistical analysis.* The incidences among the groups were compared using chi-square test or Fisher's extract probability test with the GraphPad Instat Software (version 3.05; GraphPad software Inc., San Diego, CA, USA). Other measurements expressing mean±standard deviation (SD) were statistically analyzed using Tukey-Kramer multiple comparison post test (GraphPad Instat version 3.05). Differences were considered statistically significant at $p<0.05$.

## Results

*General observation.* Body weight gains during the study were comparable among the groups (data not shown). At sacrifice, the mean weights of body, liver, and lungs and

Table I. *Mean body, liver, relative liver and lung weights, and mean colon length.*

| Treatment | No. of mice | Body weight (g) | Liver weight (g) | % Liver weight (g) | Lung weight (g) | Colon length |
|---|---|---|---|---|---|---|
| NNK+AOM+DSS | 20 | 27.5±3.6 | 1.46±0.20 | 5.31±0.32 | 0.43±0.07 | 13.7±1.0 |
| AOM+DSS | 10 | 28.1±1.5 | 1.59±0.13 | 5.66±0.35 | 0.52±0.08 | 15.1±0.6 |
| NNK+DSS | 10 | 27.7±1.2 | 1.58±0.17 | 5.70±0.49 | 0.53±0.07 | 15.2±1.0 |
| NNK alone | 5 | 30.9±1.8 | 1.72±0.18 | 5.56±0.39 | 0.56±0.07 | 16.0±0.6 |
| AOM alone | 5 | 28.4±1.8 | 1.47±0.15 | 5.19±0.54 | 0.50±0.06 | 16.1±0.6 |
| DSS alone | 5 | 28.1±1.0 | 1.53±0.09 | 5.44±0.31 | 0.45±0.04 | 15.8±0.3 |
| Untreated | 5 | 29.4±1.5 | 1.57±0.07 | 5.37±0.38 | 0.49±0.03 | 16.3±0.2 |

Data values are means±SD. NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; AOM, azoxymethane; DSS, dextran sulfate sodium.

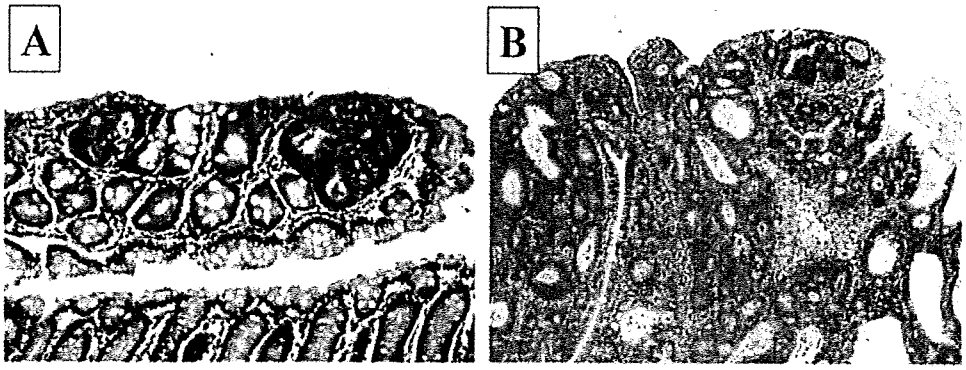


Figure 2. *Colonic neoplasms developed in mice from Group 1 that received NNK+AOM+DSS. A, tubular adenoma and B, tubular adenocarcinoma. H & E stain. Original magnification, (A) ×100 and (B) ×40.*

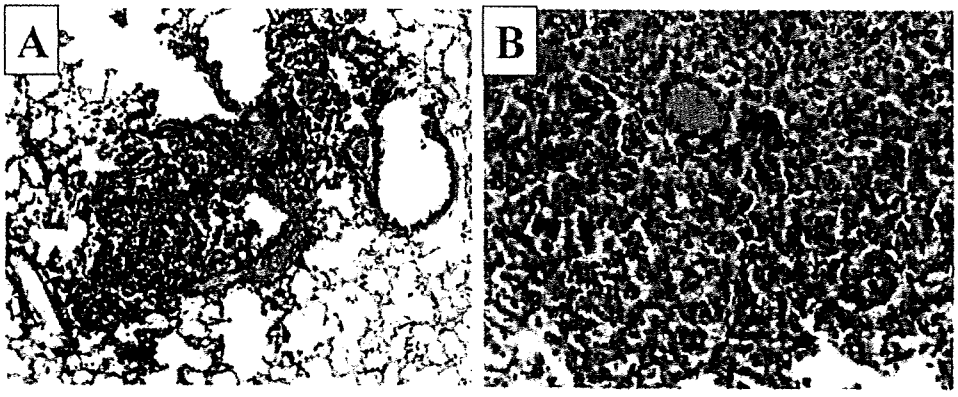


Figure 3. *Lung proliferative lesions developed in mice from Group 1 that received NNK+AOM+DSS. A, alveolar hyperplasia and B, adenoma. H & E stain, Original magnification, (A) ×40 and (B) ×100.*

colon lengths did not significantly differ among the groups, as shown in Table I.

*Effects of NNK on colon carcinogenesis.* Colonic tumors histologically diagnosed as adenoma (AD, Figure 2A) and/or adenocarcinoma (ADC, Figure 2B) developed in Groups 1 through 3, but not in Groups 4-7. Data on the incidence and multiplicity of colonic tumors are summarized in Table II. The incidence (80%) and multiplicity (4.00±3.58) of colonic tumors were the highest in Group 1, followed by

Table II. *Incidences and multiplicity of colonic tumors.*

| Treatment | No. of mice | Incidence | | | Multiplicity (no. of tumors/colon)[a] | | |
|---|---|---|---|---|---|---|---|
| | | AD | ADC | Total | AD | ADC | Total |
| NNK+AOM+DSS | 20 | 13[#] | 16[*,#] | 16[#] | 1.80±1.88[##] | 2.20±1.88[**,##] | 4.00±3.58[**,##] |
| AOM+DSS | 10 | 4 | 4 | 5 | 0.60±0.97 | 0.60±0.84 | 1.20±1.69 |
| NNK+DSS | 10 | 1 | 0 | 1 | 0.10±0.32 | 0 | 0.10±0.32 |
| NNK alone | 5 | 0 | 0 | 0 | 0 | 0 | 0 |
| AOM alone | 5 | 0 | 0 | 0 | 0 | 0 | 0 |
| DSS alone | 5 | 0 | 0 | 0 | 0 | 0 | 0 |
| Untreated | 5 | 0 | 0 | 0 | 0 | 0 | 0 |

AD, adenoma; ADC, adenocarcinoma. [a]Data values are means±SD. NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; AOM, azoxymethane; DSS, dextran sulfate sodium. *Significantly different from the AOM+DSS group by Fisher's exact probability test ($p<0.05$). #Significantly different from the NNK+DSS group by Fisher's exact probability test ($p<0.05$). **Significantly different from the AOM+DSS group by Tukey-Kramer multiple comparison post test ($p<0.05$). ##Significantly different from the NNK+DSS group by Tukey-Kramer multiple comparison post test ($p<0.05$).

Table III. *Proliferative lesions of lung.*

| Treatment | No. of mice | Incidence | | | Multiplicity (no. of proliferative lesions/lung)[a] | | |
|---|---|---|---|---|---|---|---|
| | | HP | AD | Total | HP | AD | Total |
| NNK+AOM+DSS | 20 | 20 | 6 | 20 | 4.00±2.20 | 0.55±0.90 | 4.55±2.06 |
| AOM+DSS | 10 | 10 | 0 | 10 | 4.00±1.60 | 0 | 4.00±1.56 |
| NNK+DSS | 10 | 9 | 1 | 9 | 3.30±1.80 | 0.10±0.30 | 3.40±1.90 |
| NNK alone | 5 | 5 | 3 | 5 | 3.60±0.90 | 0.80±0.80 | 4.40±1.52 |
| AOM alone | 5 | 5 | 1 | 5 | 3.20±2.30 | 0.20±0.40 | 3.40±2.30 |
| DSS alone | 5 | 5 | 0 | 5 | 3.40±2.70 | 0 | 3.40±2.70 |
| Untreated | 5 | 5 | 0 | 5 | 4.00±1.00 | 0 | 4.00±1.00 |

HP, hyperplasia; AD, adenoma. [a]Data values are means±SD. NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; AOM, azoxymethane; DSS, dextran sulfate sodium.

Group 2 (50%, 1.20±1.69) and Group 3 (10%, 010±0.32). The incidence of colonic tumors of Group 1 was significantly greater than Group 3 ($p<0.05$), and the multiplicity of colonic neoplasms developed in Group 1 was significantly larger than Groups 2 ($p<0.05$) and 3 ($p<0.05$). Colonic ADC developed in Groups 1 (80% and 2.20±1.88) and 2 (40% and 0.60±0.84) with higher incidence and multiplicity in Group 1. The values were significantly greater than those of Groups 2 and 3 ($p<0.05$ for each comparison). Only one mouse of Group 3 developed a few colonic AD.

*Effects of AOM/DSS on lung proliferative lesions.* Lung proliferative lesions (hyperplasia and/or AD) developed in all mice in all groups. Data of the incidence and multiplicity of lung proliferative lesions are presented in Table III. Lung alveolar hyperplasia (Figure 3A) developed in all mice examined. The incidence and multiplicity of lung hyperplasia did not significantly differ among the groups. Lung AD (Figure 3B) developed in Groups 1, 3, 4, and 5, but not in mice of Groups 2, 6, and 7.

## Discussion

In the current study, we investigated the effects of NNK on inflammation-associated colon cancer in male A/J mice. Our findings clearly showed that NNK enhances AOM/DSS-induced colon carcinogenesis, while treatment with AOM/DSS did not influence the occurrence of lung proliferative lesions induced by NNK. Our results were similar to epidemiological reports that current and ever smokers have increased odds of high-risk adenomas (21).

In the present study, we observed that colonic adenoma developed in a mouse of Group 3 that received NNK and DSS. Our series of investigation using an AOM/DSS mouse model used in this study demonstrated that the model can be used for detecting tumor initiation (22), tumor-promoting (23, 24) and chemopreventive (25) activities of xenobiotics in the colon. Our present findings suggest that NNK has initiation activity in the inflamed colonic mucosa, although the activity was weak. NNK is one of the nitrosamines metabolized from nicotine among the many components of

tobacco smoke (26). A/J mice are highly susceptible to chemically induced preneoplasms/neoplasms in the lung (27) and colon (28). Thus, this mouse strain seems suitable for use in a combined assay to evaluate potential modifying effects of xenobiotics on carcinogenesis in these tissues.

It is well known that cigarette smoking is a risk factor for carcinogenesis in a variety of tissues, such as those of the respiratory system (29) and digestive tract (30). In the colon, current and ever smokers have 2-fold increased odds of high-risk adenoma (21). Although it remains unclear whether smoking plays pivotal roles in colon carcinogenesis, it is likely that certain tobacco constituents, such as NNK, act as weak initiators in the carcinogenesis process. In this context, a recent report (31) identifying NNK as a high affinity ligand for neuronal nicotinic acetylcholine receptor (nAChR) comprised of α7-subunits, *i.e.* α7-nAChR, and expressed in human small cell lung carcinoma and endothelial cells is of interest. Furthermore, Ye *et al.* (11) reported that NNK enhanced α7-nAChR and its receptor mRNA expression in SW1116 human colon adenocarcinoma cells through increases in nuclear factor-κB (NFκB)-DNA binding activity and COX-2 and 5-LOX expression. Moreover, NNK treatment increased the level of intracellular reactive oxygen species (ROS) in SW1116 cells (11). NNK bioactivation leads to the production of ROS (32). ROS are known to activate NF-κB, which acts as a positive regulatory element of COX-2 expression (33). These results may indicate that α7-nAChR and ROS are involved in colonic tumorigenesis enhanced by NNK. Wong *et al.* (34) also showed that NNK stimulated HT-29 human colon cancer cell proliferation *via* activating mitogenic signal transduction pathway. This may be explained by the fact that NNK stimulated cell growth and affected the cell cycle. There are a few animal studies investigating the effects of tobacco-related carcinogens on colon tumorigenesis. Cigarette smoking enhanced colitis-related adenoma formation in mice (35) and COX-2 inhibitors reduced colitis-related colon tumorigenesis in mice (36, 37). Our results are in accordance with those reported by Liu *et al.* (35).

In the current study, AOM/DSS and DSS treatment (Groups 1 and 3 *vs.* Group 4) did not affect lung tumorigenesis induced by NNK. A tobacco-specific carcinogen, NNK can be stored in mammalian organisms. Importantly, the amount of NNK used in this study is high enough such that the total estimated doses to smokers and long-term snuff-dippers are similar in magnitude to the total doses required to produce cancer in laboratory animals (38, 39). This may be explained by the fact that after being given DSS in drinking water, DSS is mainly detected in Kupffer cells of the liver and macrophages of the mesenteric lymph nodes and large intestine, but not in the lung (40). Thus, DSS would not seem to be able to promote NNK-induced lung tumorigenesis. Another explanation for a lack of modifying effects of DSS on lung tumorigenesis is that DSS did not alter CYP2A6 expression in the lung and liver, which is responsible for activation of NNK (41). AOM requires metabolic activation by CYP2E1 to exert its carcinogenic action (42). Both CYP2E1 and CYP2A6 are responsible for the metabolic activation of *N*-nitrosamines (43). Although we did not investigate the activity of CYP2A6 and CYP2E1 in the current study, it is important to investigate whether smoking and colitis affect expression of CYP2A6 and CYP2E1 in the colon and/or lung. In addition, it would be interesting to investigate the exact mechanisms, including immunomodulation (44) and cytokine expression (45), in order to determine how NNK contributes to colon tumorigenesis in the inflamed colon.

Although the effects of smoking on development of UC and CD are different (46), cigarette smoke exposure increases colitis-associated colonic adenoma formation in mice (35) and current and ever smokers have an increased risk of colonic adenoma (21). Our findings that a tobacco-specific carcinogen, NNK, influences inflammation-associated colon carcinogenesis in mice support these reports (21, 35).

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