Chemopreventive Effect of 4-[3,5-Bis(trimethylsilyl) benzamido] Benzoic Acid (TAC-101) on MNU-induced Colon Carcinogenesis in a Rat Model

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Abstract. *Purpose: 4-[3,5-Bis(trimethylsilyl)benzamido] benzoic acid (TAC-101) is a novel retinobenzoic acid derivative. The chemopreventive effect and the mechanism of action of TAC-101 were investigated using a rat chemical colon carcinogenesis model. Materials and Methods: Colon tumors were induced using intra-rectal instillation of N-methyl-N-nitrosourea (MNU) in F344 rats. These rats were divided into five groups, control, high dose (TAC-101 8 mg/kg)-long period (four weeks), high dose-short period (one week), low dose (TAC-101 0.8 mg/kg)-long period and low dose-short period. After the large bowels were resected at 20 weeks, the number of aberrant crypt foci (ACF) and tumors in the colon were counted. Proliferating cell nuclear antigen (PCNA) positive index, apoptotic index (AI) and Fas expression were also evaluated using immunohistochemistry. Results: The tumor incidence and the tumor number in the high dose-long period group were decreased in comparison to those in the other groups, but not significantly. However, the number of ACF or PCNA positive indices in the high dose-long period group was significantly decreased in comparison to that in the other groups. On the other hand, the AI and the Fas expression pattern in the tumor and the normal appearing mucosa were not changed in any of the groups. Conclusion: TAC-101 might inhibit MNU induced colon carcinogenesis via a decrease of ACF. The mechanism of this chemoprevention may be related to a reduction in cell proliferation, but is not directly associated with apoptosis.*

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Several synthetic retinoids have been created and evaluated as potential chemoprevention and chemotherapeutic agents. Retinoic acids (RAs) are multifunctional drugs that have been shown to suppress carcinogenesis in various epithelial tissues in animal model systems (1, 2), to have clinical efficacy as chemotherapeutic agents against selected malignancies (3-5), to prevent the development of multiple primary tumors (6-8). Moreover, successful retinoid treatment of premalignant lesions such as oral leukoplakia (6), cervical dysplasia (7) and xeroderma pigmentosum (8) has demonstrated their clinical chemopreventive potential. Retinoids have also clinically reduced secondary malignancies in the head and neck (9), the liver (10), the lung (11) and in the breast (12). Preclinical studies have also indicated that several natural and synthetic retinoids may be potential chemopreventive agents against colon carcinogenesis (13-15). The mechanism of the antitumor effects of RAs is associated with effects on the proliferation, differentiation, apoptosis and angiogenesis of several types of cancer cells (16-19). For example, all-trans-retinoic acid (ATRA) has been clinically used as a therapeutic agent for acute premyelocytic leukemia (APL) because of its effects on differentiation and apoptosis (5). On the other hand, the chemopreventive mechanism of RAs on carcinogenesis seems to be related to their reduction of cell proliferation and enhancement of differentiation in several organs (16, 17). However, the details of the mechanisms of such chemoprevention still remain unclear.

Non steroidal anti-inflammatory drugs (NSAIDs) suppress colon carcinogenesis at the adenoma stage *via* cyclooxygenase

(COX)-2 which is a major molecular target for this action and is mainly expressed in intestinal cells (20, 21). Moreover, in the large bowel, the cancer-preventive activity of aspirin and NSAIDs is principally associated with their ability to block the generation of prostaglandin (PG) E2 by COX-2 (22, 23). β-carotene also decreases COX-2 expression and PGE2 production in colon cancer cells (24). On the other hand, a previous report indicated that TAC-101, 4-[3,5-Bis(trimethylsilyl)benzamido] benzoic acid, a synthetic retinoid, inhibits the interleukin (IL)-1β-induced PGE2 production by human osteoblast-like MG-63 cells through the suppression of COX-2 mRNA expression (25).

TAC-101 is a novel benzoic acid derivative and it is possible that TAC-101 might be useful for chemoprevention against colon carcinogenesis. Several reports have demonstrated the life-prolonging effect (26-31), the antitumor effect and the antimetastatic effect (26-30, 32-35) of TAC-101 *in vivo* and *in vitro*. These effects of TAC-101 might be associated with the inhibition of cell proliferation (29, 32, 34, 36, 37), angiogenesis (32, 35, 37, 38), and invasion (26, 29), the induction of apoptosis (26, 29, 31, 33, 36, 39) and the enhancement of cell differentiation (40, 41). TAC-101 also may suppress liver metastasis by the induction of an apoptotic mechanism in cancer cells and possibly by controlling the transcriptional activity of activator protein (AP)-1 (30).

On the other hand, the chemopreventive effects of TAC-101 against colon carcinogenesis have not been evaluated. Therefore, this study examined whether TAC-101 could prevent colon carcinogenesis in a rat model.

Materials and Methods

Animals, diet and materials. Male 7-week-old F344/DuCrj rats were purchased from Charles River Japan Inc. (Atsugi, Japan). The rats were maintained according to the Institutional Animal Care Guidelines. They were housed in 3-per-rack-mounted wire cages and given tap water *ad libitum*. The temperature (20-22˚C), humidity (45-55%) and light (12 hour light/dark cycle) were constantly controlled.

TAC-101 was synthesized and kindly donated by Taiho Pharmaceutical Co., Ltd. (Hanno, Japan). The TAC-101 was suspended in 0.5% hydroxypropoxyl methyl cellulose. The TAC-101 was orally administered for 5 consecutive days a week. N-methyl-N-nitrosourea (MNU) was purchased from Nakarai Chemicals, Ltd. (Kyoto, Japan). A 0.4 % distilled water solution of MNU was freshly prepared each time before use.

Rat colon carcinogenesis model. The rats of all groups received an intra-rectal instillation of NMU solution (0.5 ml, 2 mg/rat) three times a week for 4 weeks (42). Thirty-eight rats were assigned to one of five regimens and administered high (8 mg/kg) or low (0.8 mg/kg) dose TAC-101 for a long (four weeks) or short (one week) period. NMU and TAC-101 were started on day 1. The control group (9 rats) were orally administered only the vehicle for four weeks. The treated groups were high-long (8 rats), high-short (8 rats), low-long (6 rats) and low-short (7 rats). The large bowels were resected at 20 weeks after the start of NMU and TAC-101. After the determination of aberrant crypt foci (ACF) and the tumors in each resected colon, these colons were embedded in paraffin for immunohistochemical staining.

Determination of aberrant crypt foci (ACF). The resected colon segments were fixed in 10% buffered formalin for 24 hour, stained with 0.2% methylene blue and examined under 40x magnification (13). ACFs were distinguished from the surrounding normal crypts by their increased size, noticeably increased distance from the lamina to the basal surface of the cells and the easily discernible pericryptal zone.

Immunohistochemical staining for PCNA. After deparaffinization, dehydration and the blocking of endogenous peroxidase activity, the sections were incubated in 10% normal goat serum to reduce nonspecific antibody binding. Mouse anti-proliferating cell nuclear antigen (PCNA) antibody (1:200; PC-10, Dako Japan, Kyoto, Japan) was used for 60 minutes at room temperature as the primary antibody (43). After treatment with EnVision+ reagent (EnVision+ system, Dako, Copenhagen, Denmark) for 30 minutes (44), the sections were incubated in PBS containing diaminobenzidine and 1% hydrogen peroxidase for 10 minutes, counterstained with hematoxylin and mounted.

For determination of the PCNA-positive index, 10 full-length normal appearing crypts of each colon were examined. The number of PCNA positively stained nuclei in each crypt column was recorded. The PCNA-positive index (number of positive stained nuclei ×100/total number of nuclei counted) was then calculated (45).

Immunohistochemical staining for single stranded (ss) DNA. The apoptotic cells in the tumor or normal appearing mucosa were stained by immunohistochemistry for single-stranded (ss) DNA (46). After deparaffinization, dehydration and the blocking of endogenous peroxidase activity, the sections were incubated in 10% normal bovine serum for 5 minutes, followed by incubation for 1 hour with a rabbit polyclonal antibody against ssDNA (1:100; Dako Japan). After biotinylated goat anti-rabbit IgG (1:500; Dako LSAB (Labeled StreptAbidine-Biotin) kit; Dako Japan) was used, 0.02% diaminobenzidine and 1% hydrogen peroxidase in PBS were used as the substrate. The sections were counterstained with hematoxylin.

Determination of apoptotic index (AI). The AI was expressed as the number of positively ssDNA staining tumor cells among 1000 tumor cells (46). Five representative areas of a section, without necrosis, were selected by light microscopy using 200-fold magnification. Positively staining tumor cells with the morphological characteristics of apoptosis were identified using standard criteria (47).

Immunohistochemical staining of Fas. After deparaffinization, dehydration and the blocking of endogenous peroxidase activity, the sections were incubated in 10% normal goat serum to reduce nonspecific antibody binding. The sections were incubated with FAS (C-20)-G (1:500: Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) rabbit polyclonal antibody (48) for 60 minutes at room temperature. After treatment with EnVision+ reagent for 30 minutes (44), the sections were then incubated with peroxidase-labeled streptavidin reagent (1:500; LSAB kit/HRP (horseradish peroxidase), Nichirei Corporation, Tokyo, Japan) for 60 minutes. Finally, the slides

Table I. *Body and organ weights after TAC-101 administration in MNU treated F344 rats at 20 weeks.*

Group	n	body (g) (mean±S.D.)	liver (g) (mean±S.D.)	spleen (g) (mean±S.D.)	kidney (g) (mean±S.D.)
Control	9	175.7±11.9	5.00±0.72	0.44±0.03	0.67±0.06
High-long	8	176.4±6.2	5.07±0.44	0.43±0.04	0.72±0.03
High-short	8	176.1±5.0	5.25±0.37	0.42±0.02	0.68±0.04
Low-long	6	178.2±5.2	5.17±0.07	0.48±0.06	0.71±0.03
Low-short	7	175.3±12.0	5.13±0.36	0.42±0.01	0.70±0.11

High: 8 mg/kg, low: 0.8 mg/kg TAC-101 dose; long: 4weeks, short: 1 week administration. S.D.: standard deviation.

Table II. *Colonic tumors and aberrant crypt foci (ACF) frequency after TAC-101 administration in MNU treated F344 rats.*

Group	n	Colonic tumors		ACF (mean±S.D.)
		Tumor incidence	No. of tumor (mean±S.D.)	
Control	9	7/9 (78%)	1.00±0.71	19.7±3.94
High-long	8	3/8 (38%)	0.50±0.76	4.9±1.81*
High-short	8	5/8 (63%)	1.13±0.99	23.4±3.54
Low-long	6	4/6 (67%)	1.00±1.10	20.2±2.32
Low-short	7	5/7 (71%)	1.00±0.82	17.9±3.85

High: 8 mg/kg, low: 0.8 mg/kg TAC-101 dose; long: 4weeks, short: 1 week administration. S.D.: standard deviation. *$p<0.001$ (Student's *t*-test) *vs*. others.

were incubated in PBS containing diaminobenzidine and 1% hydrogen peroxidase for 10 minutes, then were counterstained with hematoxylin and mounted.

Statistical methods. All the data are expressed as the mean±SD and were statistically analyzed using Student's *t*-test and the regression theory, as appropriate. The relationships between the parameters also were assessed statistically by the Chi-square test using Stat View-J statistical package (version 5.0, SAS institute. Inc., Cary, NC, USA). Statistical significance was established at the $p<0.05$ level.

Results

Table I shows that the weight of the rat bodies and organs such as the liver, spleen and kidney were not significantly different in any of the groups.

Table II indicates that the tumor incidence and tumor number in the high dose-long period group were decreased in comparison to that in the other groups, but the difference was not significant. The number of ACF in the high dose-long period group was significantly decreased in comparison to that in the other groups ($p<0.001$) (Table II).

PCNA positive cells were observed mainly in the proliferating and transitional zone of the crypts (Figure 1). In the high dose-long period group, TAC-101 significantly decreased the PCNA positive index of the normal mucosa in comparison to that of the other groups (Table III). On the other hand, TAC-101 did not affecte the PCNA positive index of the tumor in any of the groups (data not shown).

The AI in the colorectal tumors of the rats showed no difference in any group (Table III). Moreover, the AI of the normal mucosa showed no difference in any group (data not shown).

The Fas expression pattern was also the same in all the groups (Figure 2). Very few of the tumor cells and normal appearing mucosal cells were stained by the Fas antibody. Fas positive cells were mainly observed in the tumor stroma.

Table III. *PCNA labeling index in normal mucosa and apoptotic index (AI) of the tumor after TAC-101 administration in MNU treated F344 rats.*

Group	n	PCNA positive index(%) (mean±S.D.)	AI (cells/1000) (mean±S.D.)
Control	9	71.4±9.5	6.40±1.09
High-long	8	39.6±3.6*	6.45±0.39
High-short	8	50.6±1.2	6.89±1.07
Low-long	6	63.4±4.5	6.84±0.23
Low-short	7	57.3±2.1	7.00±0.00

High: 8 mg/kg, low: 0.8 mg/kg TAC-101 dose; long: 4weeks, short: 1 week administration. S.D.: standard deviation. *$p<0.01$ (Student's *t*-test) *vs*. others.

Discussion

Each retinoid shows specific binding affinity to retinoic acid receptors (RARs) or retinoid X receptors (RXRs) and has particular effects in chemoprevention and chemotherapy (49-52). TAC-101 has a strong binding affinity for RARα and a weak binding affinity for RARβ (26). The pattern of TAC-101 binding affinity may be specific and different from the other synthetic RAs. Therefore, we speculate that the chemopreventive mechanism of TAC-101 may be different from other synthetic RAs.

The suppression of colonic ACF formation has been established as a short-term assay to screen candidate compounds for chemopreventive activity in colon carcinogenesis studies in rats (13). *All-trans*-RA and 13-*cis*-RA have been reported to reduce the yield of ACF by AOM (13). Moreover, 9-*cis*-RA, 2-CPR (2-(carboxyphenyl) retinamide), 4-HPR (4-hydroxyphenyl)retinamide) and β-Ionone were highly effective in decreasing the yield of colon tumors and ACF (14, 15, 53) in an AOM-induced colon

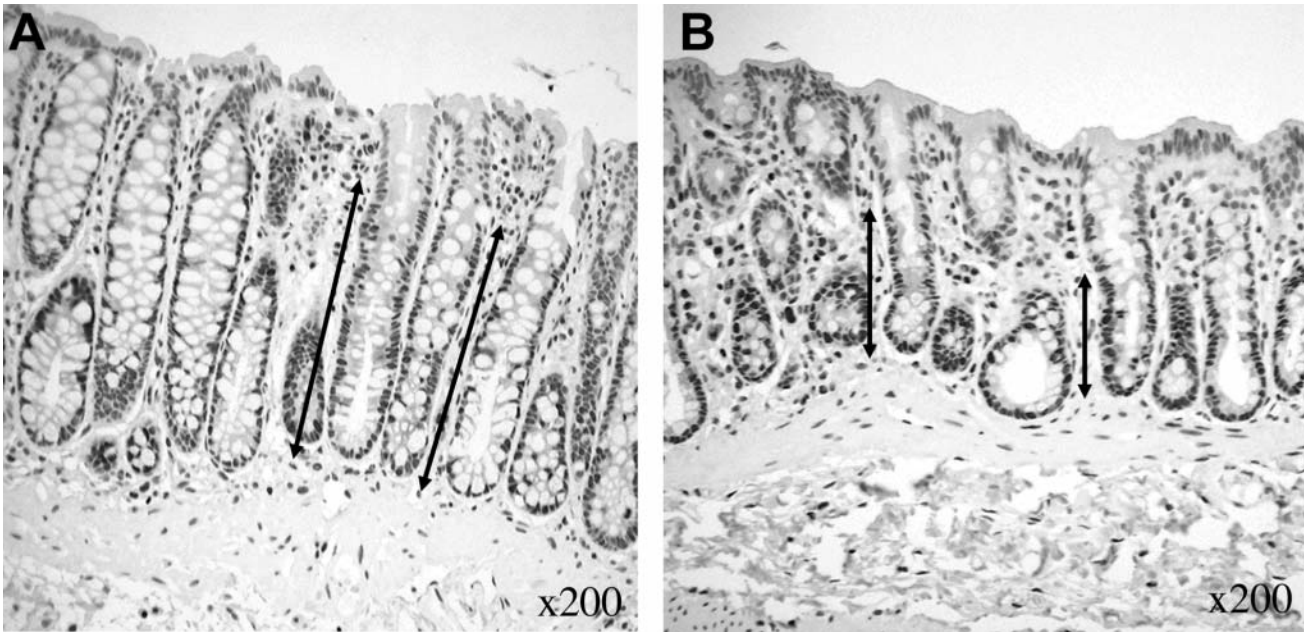

Figure 1. *Immunohistochemical staining of PCNA in colon sections. PCNA positive cells (arrow bar) in the control group (A) and the high-long group (B).*

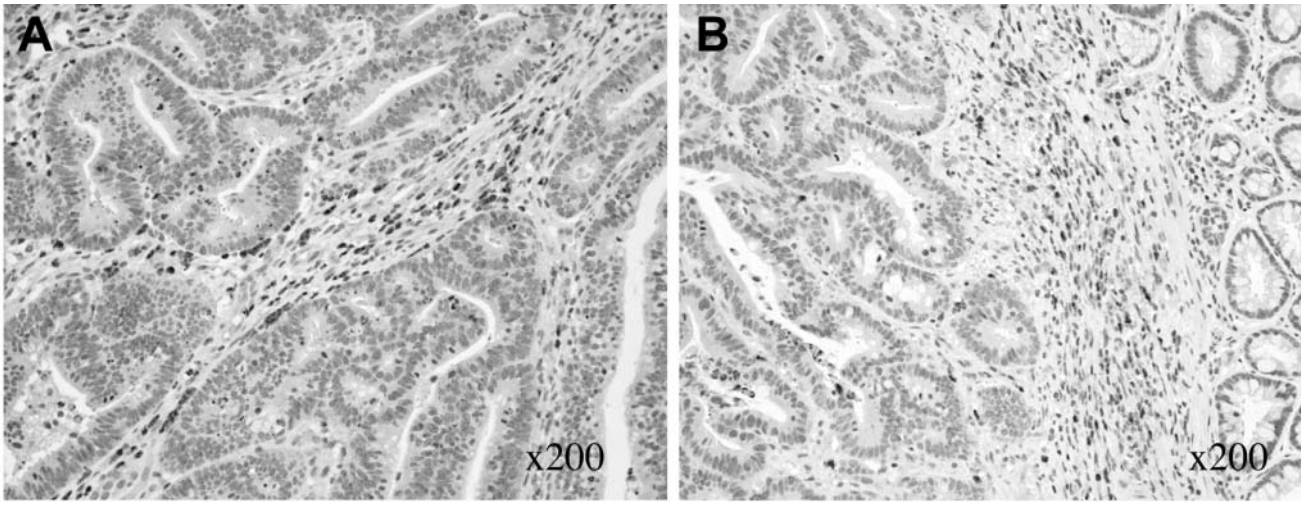

Figure 2. *Immunohistochemical staining of Fas. Control group (A) and the high-long group (B).*

carcinogenesis model. Moreover, some of these RAs reduced the PCNA positive index, reflecting cell growth, on normal crypts (53). In the present study, TAC-101 also decreased the PCNA positive index in the normal appearing mucosa and the number of ACFs.

TAC-101 did not affect the AI and Fas expression pattern in the colorectal tumors or the normal appearing mucosa of the rats in the present study (Table III, Figure 2). On the other hand, TAC-101 significantly increased the AI and the expression of Fas in metastatic tumors in a hepatic metastatic model (36). These discrepancies may be based on the difference between the carcinogenic state and metastatic state of the cancer. If TAC-101 had been administered to the rats during both the initiation phase and the promotion/progression phase in this study, the AI and the expression of Fas in the colonic tumors or normal appearing mucosa might have shown different results. Further examination will be needed to explain these points.

In general, chemoprevention must be safe. In a rat experimental model, the oral administration of TAC-101 (8 mg/kg) for 5 consecutive days a week for four weeks resulted in a significant inhibition of hepatic metastasis without weight loss of the rats and did not markedly affect the histological appearance of the tissue of the liver, kidney, lung or heart (36). Moreover, the present study showed that the weight of the rat bodies and organs such as the liver, spleen and kidney were not significantly different between the groups (Table I). The rats exhibited no sudden death or necrosis of the extremities associated with the daily oral administration of TAC-101 in these studies. Therefore, the chemopreventive use of TAC-101 appears to be safe and feasible.

In conclusion, TAC-101 is a potent chemopreventive in a rat colon carcinogenesis model. TAC-101 might inhibit MNU-induced colon carcinogenesis by decreasing the number of ACFs. The mechanism of this chemoprevention may be associated with the reduction in cell proliferation but is not directly associated with apoptosis. TAC-101 may be a good candidate for use in the chemoprevention of the colon cancer.

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