

Dietary Supplementation with Silymarin Inhibits 3,2'-Dimethyl-4-Aminobiphenyl – Induced Prostate Carcinogenesis in Male F344 Rats

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Abstract Purpose: Silymarin has been shown to be a potent anticarcinogenic agent. Here, we investigated the modifying effects of dietary feeding with a naturally occurring polyphenolic antioxidant flavonoid silymarin on 3,2'-dimethyl-4-aminobiphenyl (DMAB) – induced prostatic carcinogenesis in male F344 rats.

Experimental Design: Male F344 rats were given s.c. injections of DMAB (25 mg/kg body weight) every other week for 20 weeks. They also received the experimental diet containing 100 or 500 ppm silymarin for 40 weeks, starting 1 week after the last dosing of DMAB. All of the rats were sacrificed 60 weeks after the start of the experiment. Histopathology and immunohistochemistry for proliferative cell nuclear antigen, cyclin D1, and apoptotic indices were done in the prostatic lesions, including invasive adenocarcinomas, intraepithelial neoplasms, and nonlesional glands.

Results: Dietary feeding with 500 ppm silymarin significantly inhibited the incidence of prostatic adenocarcinoma when compared with the DMAB-alone group (17.6% versus 50.0%, $P < 0.05$). The proliferative cell nuclear antigen – and cyclin D1 – positive indices in adenocarcinomas, prostatic intraepithelial neoplasm, and nonlesional glands in rats treated with DMAB and silymarin were slightly lower than that of the DMAB-alone group. Also, dietary administration of silymarin increased apoptotic index in prostatic adenocarcinoma by measuring immunohistochemically positive nuclei for ssDNA.

Conclusions: Our results indicate that silymarin exerts chemopreventive ability against chemically induced prostatic carcinogenesis through apoptosis induction and modification of cell proliferation.

Prostate cancer is the most common type of cancer found in older men and the leading cause of cancer mortality in men (1). In Japan, the incidence and mortality rates of this malignancy are lower compared with Western populations (2), but they have gradually increased (3). Furthermore, migrant studies have shown that the incidence of prostate cancer increases generation by generation after immigration in Japanese-

Americans (4). These observations strongly suggest that the wide disparity in prostate cancer incidence worldwide is attributable to dietary habits, among which are a regimen rich in several flavonoids and isoflavones that inhibits the progression of prostate cancer by modulating epigenetic events (5). It is, therefore, necessary to intensify our efforts to better understand this disease and develop novel approaches for its prevention and treatment.

Silymarin, the collective name for an extract from the milk thistle [*Silybum marianum* (L.) Gaertner], is a naturally occurring polyphenolic flavonoid antioxidant. It is composed mainly of silibinin (~80%, w/w; also called silybin, silibin, or sibilinin) with smaller amounts of other stereoisomers (isosilybin, dihydrosilybin, silydianin, and silychristin, etc.; ref. 6). Silymarin has strong antioxidative properties and is able to scavenge both free radicals and reactive oxygen species (7, 8). In Europe, for over 20 years, silymarin, as an antihepatotoxic, is used clinically for the treatment of alcoholic liver disease (9, 10). In recent years, silymarin has also been used in Asia as a therapeutic agent for liver diseases (6). Silymarin is well tolerated and largely free of adverse effects (6, 11). Silymarin acts as a potent anticarcinogenic agent against *in vitro* and *in vivo* carcinogenesis experiments (12–14). Silymarin and silibinin, which is the major active constituent of silymarin, can inhibit the growth of human prostate carcinoma LNCaP, PC-3, and DU145 cells in culture (15–17). Moreover, silymarin and

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silibinin inhibit cell growth and induce apoptosis in rat prostate cancer cell lines (18). However, chemoprevention studies using silymarin in rodent were limited to skin (13, 19). Silymarin inhibits tumor promoter–caused induction of ornithine decarboxylase activity and mRNA expression in mouse epidermis (20). Silymarin inhibits mRNA expression of endogenous tumor promoter tumor necrosis factor- α (21). More recently, silymarin has been reported to inhibit activation of erbB1 signaling, induce cyclin-dependent kinase inhibitors, G₁ arrest, and cause complete inhibition of growth of human prostate carcinoma DU145 cells (17). Also, silymarin, at lower nontoxic concentrations, can inhibit transformation in cultured rat tracheal epithelial cells treated with benzo(a)pyrene (22). These findings led us to evaluate the possible suppressing effects of dietary silymarin on the occurrence of chemically induced neoplasms in organs other than skin of rodents. We recently have found the inhibitory effects of dietary administration of silymarin against rat tongue (23), mouse urinary bladder (24), and rat colon (25) carcinogenesis.

In the current study, we investigated the effects of silymarin on 3,2'-dimethyl-4-aminobiphenyl (DMAB)–initiated prostate carcinogenesis in male F344 rats. Also, the modulatory effects of the silymarin on the proliferating cell nuclear antigen (PCNA), cyclin D1, and apoptotic indices were immunohistochemically investigated in the prostatic lesions induced by DMAB.

Materials and Methods

Animals, chemicals, and diets. Four-week-old male F344 rats (Charles River Japan, Inc., Kanagawa, Japan) were used. The animals were maintained in the Kanazawa Medical University Animal Facility according to the Institutional Animal Care Guidelines. All animals were housed in polycarbonate cages (three or four rats per cage) under controlled conditions of humidity ($50 \pm 10\%$), lighting (12-hour light/dark cycle), and temperature ($23 \pm 2^\circ\text{C}$). They have free access to drinking water (ion exchange water) and a basal diet, CRF-1 (Oriental Yeast, Co., Ltd., Tokyo, Japan) from which soy constituents were eliminated throughout the study. Animals were quarantined for 7 days and randomized by body weight into experimental and control groups. DMAB and silymarin were obtained from Sigma-Aldrich Japan, K.K. (Tokyo, Japan). The experimental diet containing silymarin were prepared by Oriental Yeast by adding test chemicals to soy protein-free CRF-1.

Experimental procedure. A total of 68 male F344 rats were divided into nine experimental and control groups. The animals in groups 1 through 3 were given DMAB dissolved in DMSO, s.c., at a dose of 25 mg/kg body weight every other week for 20 weeks. DMAB injection was done between 10:00 a.m. and 11:00 a.m. From 1 week

after the last injection of DMAB, group 1 was given the basal diet without silymarin, groups 2 and 3 received silymarin-containing diets (100 ppm for group 2 and 500 ppm for group 3), and group 4 was fed the diet containing 500 ppm silymarin for 40 weeks. Group 5 served as an untreated control. The doses of the test compounds were selected based on previous studies (23). All rats were sacrificed at week 60 by ether overdose to assess the pathologic lesions in all organs, including prostate. At autopsy, all organs were carefully inspected and all macroscopic pathologic findings were recorded. All grossly abnormal lesions in any tissue and the organs, such as accessory sex organs including prostate, liver, kidney, lung, and heart, were fixed in 10% phosphate-buffered formalin for 2 weeks. As for the accessory sex organs, two sagittal slices of the ventral prostate, two sagittal slices of the dorsolateral prostate, which included the urethra, and three transverse slices from each side of the seminal vesicles, which included the anterior prostate, were made and embedded in paraffin. They were then sectioned and stained with H&E for histopathologic diagnosis. The prostatic lesions, including prostatic intraepithelial neoplasm (PIN; ref. 26), were histopathologically diagnosed. The diagnosis of PIN was based on the criteria described by Bostwick and Brawer (27): PIN shows the morphologic continuum of cellular proliferations with nuclear atypia that occur within prostatic ducts, ductules, or acini, and is enclosed by a basement membrane. Several architectural patterns, such as flat, tufting, microcapillary, or cribriform, could be seen.

Immunohistochemistry. For the determination of cell proliferation and cell cycle activity of the epithelial cells, PCNA and cyclin D1 immunohistochemistry was done according to the method described previously with some modifications (28, 29). Apoptotic index was also evaluated by immunohistochemistry for ssDNA (28). Immunohistochemistry was done using a stain system kit (DAKO LSAB 2 kit/HRP, DAKO Japan Co., Ltd., Kyoto, Japan). The sections (3 μm in thickness) made from paraffin-embedded tissues were deparaffinized; they were treated sequentially with 0.3% H₂O₂, normal goat serum or horse serum, and first antibodies. A mouse anti-PCNA antibody (1:100 dilution; DAKO Japan), a rabbit polyclonal anti-cyclin D1 antibody (1:3,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), and a rabbit polyclonal antibody against ssDNA (1:300; DAKO Japan) were applied to the sections according to the manufacturer's protocol (DAKO LSAB 2 kit/HRP, DAKO Japan). All incubation steps were carried out for 15 minutes at 37°C. The chromogen used was 3,3'-diaminobenzidine tetrahydrochloride. The tissues were lightly counterstained with hematoxylin to facilitate orientation. Negative controls were stained without the first antibodies. The numbers of cells with positive reactivity for PCNA, cyclin D1, and ssDNA antibody were counted in a total of 3 \times 100 cells in three different areas of the tumors, PIN, and nonlesional areas, and expressed as percentage (mean \pm SD).

Statistical evaluation. Where applicable, data were analyzed using Fisher's exact probability test, Student's *t* test, or Welch *t* test with *P* < 0.05 as the criterion of significance.

Table 1. Intakes of food and test chemical

Group no.	Treatment	No. rats examined	Daily intake		Total intake of test chemical (mg)
			Food (g/d/rat)	Test chemical (mg/d/rat)	
1	DMAB	18	16.1 \pm 2.5*	—	—
2	DMAB \rightarrow 100 ppm silymarin	17	16.0 \pm 2.6	1.60	44.8
3	DMAB \rightarrow 500 ppm silymarin	17	16.4 \pm 2.3	8.20	2,296
4	500 ppm silymarin	8	16.3 \pm 2.2	8.15	2,282
5	None	8	15.8 \pm 1.7	—	—

*Mean \pm SD.

Table 2. Body, liver, prostate, and testicular weights at the end of the study

Group no.	Treatment	No. rats examined	Body weight (g)	Liver weight (g)	Prostate weight (g)	Testes weight (g)
1	DMAB	18	384.8 ± 24.4*	11.82 ± 1.25	3.19 ± 0.61	2.88 ± 0.66
2	DMAB → 100 ppm silymarin	17	386.0 ± 21.3	12.30 ± 0.93	3.18 ± 0.59	2.80 ± 0.55
3	DMAB → 500 ppm silymarin	17	388.7 ± 16.1	12.42 ± 1.00	3.13 ± 0.67	2.79 ± 0.48
4	500 ppm silymarin	8	401.1 ± 11.8	11.76 ± 1.16	3.13 ± 0.45	2.98 ± 0.64
5	None	8	383.9 ± 20.9	12.42 ± 1.19	3.20 ± 0.62	3.03 ± 0.37

*Means ± SD.

Results

General observation. All animals remained healthy throughout the experimental period. During the study, no clinical signs of toxicity were present in any groups. Histologically, there were no pathologic alteration suggesting toxicity of silymarin in the liver, kidneys, lung, and heart. Food consumption (g/d/rat) did not significantly differ among the groups, as shown in Table 1. Estimated intakes of test chemicals were well correlated with doses applied (Table 1). Body, liver, prostate, and testicular weights in all groups at the end of the study are shown in Table 2. The mean body weights, liver, prostate, and bilateral testicular weights did not significantly differ among the groups.

Incidence of neoplasms of prostate and other organs. Table 3 summarizes the data on the incidence of neoplasms of prostate and other tissues. DMAB exposure could induce PIN and adenocarcinomas (Fig. 1A and B) in the ventral lobe of the prostate. Such lesions were not found in other lobes of the prostate and seminal vesicle. Treatment of DMAB alone (group 1) produced 50.0% incidence of well-differentiated prostatic adenocarcinoma (Fig. 1A). The incidence of prostatic adenocarcinoma (Fig. 1B) in group 3 that received DMAB and 500 ppm silymarin (17.6%) was significantly lower than in group 1 (50.0%, $P < 0.05$). Also, feeding with 100 ppm silymarin after DMAB administration (group 2) caused a reduction of incidence of prostatic adenocarcinoma (29.4%), but there was no statistical significance different from group 1. The incidences of prostatic PIN were 50.0% in group 1, 23.5% in group 2, and 64.7% in group 3. These values also

did not show statistically significance among the groups 1 through 3. In other organs, a few neoplasms, such as colonic adenocarcinoma, s.c. malignant fibrous histiocytoma, and ear duct squamous cell carcinoma, were noted in a few rats of groups 1 to 3. The incidences of these tumors were not statistically significant among the groups. No prostatic neoplasms were found in groups 4 (500 ppm silymarin alone) and 5 (no treatment).

Immunohistochemical findings. The data on PCNA- (Fig. 1C and D), cyclin D1- (Fig. 1E and F), and apoptosis-positive cells (Fig. 1G and H) in the prostatic lesions are indicated in Table 4. The mean PCNA labeling indices of adenocarcinoma found in group 3 receiving DMAB and 500 ppm silymarin (6.3 ± 1.5) were significantly lower than in group 1 (10.0 ± 2.4 , $P < 0.05$). The PCNA labeling indices of PIN in groups 2 (6.8 ± 1.7) and 3 (6.8 ± 2.2) were lower than that of group 1 (8.8 ± 2.9), but the differences were not statistically significant. As for the histologically normal prostatic glands, the PCNA-labeling indices of all groups were comparable. The mean cyclin D1 labeling indices of adenocarcinoma found in groups 2 and 3 were significantly lower than in group 1 ($P < 0.05$ or $P < 0.01$). The cyclin D1 labeling indices of PIN in groups 2 and 3 were slightly lower than that of group 1, but the differences were not statistically significant. As for the histologically normal prostatic glands, the cyclin D1 labeling indices of all groups were comparable. The apoptotic index of PIN and adenocarcinoma in group 3 was statistically greater than that of group 1 ($P < 0.05$ and $P < 0.02$, respectively). On the other hand, the apoptotic index of PIN and adenocarcinoma

Table 3. Incidence of pathologic lesions

Group no.	Treatment	No. rats examined	No. rats with incidence		
			Prostate		Others*
			PIN	Adenocarcinoma	
1	DMAB	18	9 (50.0%)	9 (50%)	4 (22.2%)
2	DMAB → 100 ppm silymarin	17	4 (23.5%)	5 (29.4%)	4 (23.5%)
3	DMAB → 500 ppm silymarin	17	11 (64.7%)	3† (17.6%)	2 (11.8%)
4	500 ppm silymarin	8	0 (0%)	0 (0%)	0 (0%)
5	None	8	0 (0%)	0 (0%)	0 (0%)

*Colonic adenocarcinoma, s.c. malignant fibrous histiocytoma, and ear duct squamous cell carcinoma.

†Significantly different from group 1 by Fisher's exact probability test ($P < 0.05$).

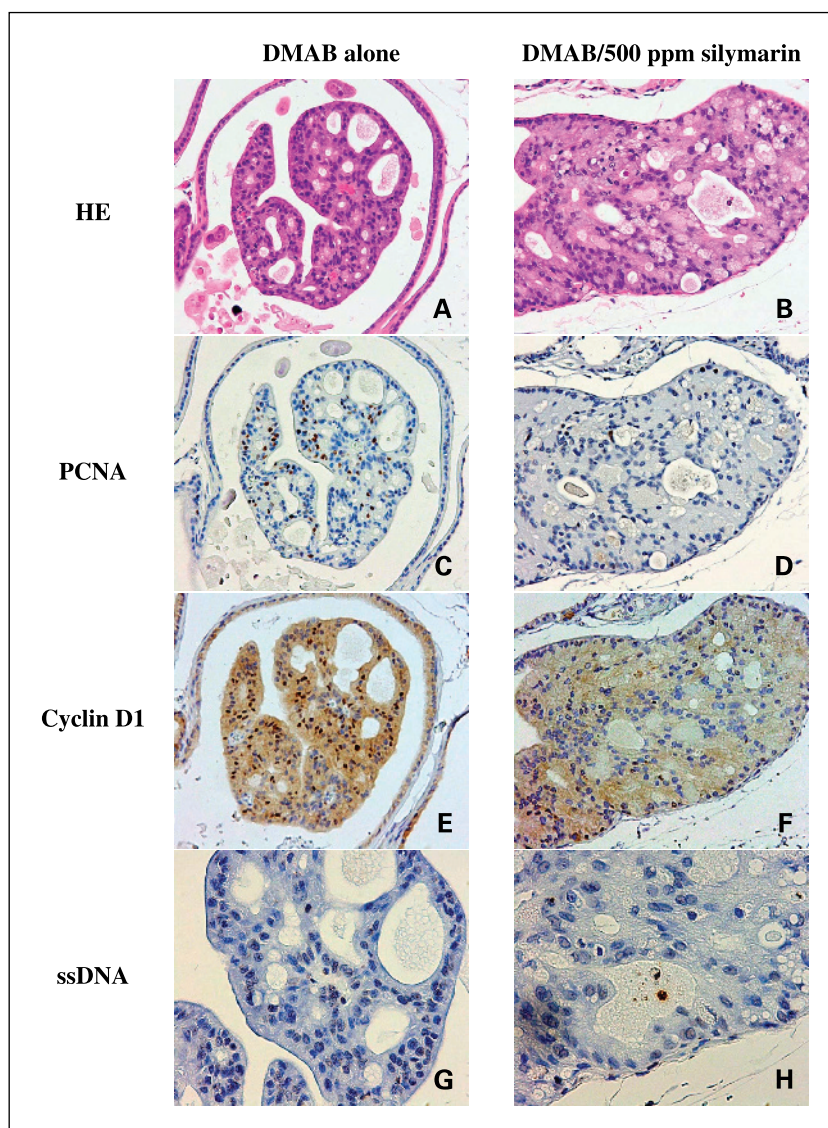


Fig. 1. Histopathology of adenocarcinomas and their immunohistochemistry of PCNA, cyclin D1, and ssDNA. An adenocarcinoma (A, C, E, G) from a rat given DMAB alone (group 1) and that (B, D, F, H) from a rat given DMAB and 500 ppm silymarin (group 3). H&E stain (A, B) and immunohistochemistry for PCNA (C, D), cyclin D1 (E, F), and ssDNA (G, H). Original magnification, $\times 20$ (A-F) and $\times 40$ (G, H).

in group 2 was lower than that of group 1, but the differences were not statistically significant. As for the histologically normal prostatic glands, the apoptotic indices of all groups were comparable.

Discussion

In the present study, dietary administration of 500 ppm silymarin during the promotion phase of DMAB-induced prostatic carcinogenesis significantly inhibited the incidence of prostatic adenocarcinoma. Silymarin is known to inhibit chemically induced carcinogenesis in skin (13), tongue (23), urinary bladder (24), and colon (25). Furthermore, Singh et al. (30) reported that the preventive and therapeutic efficacy of dietary feeding of silibinin on human prostate carcinoma DU145 tumor xenograft in athymic nude mice. These results indicate that silymarin might be a candidate chemopreventive agent against carcinogenesis in multiple organs including prostate.

Several mechanisms by which chemopreventive agents exert their inhibitory effects on tumorigenesis could be considered.

Cell proliferation plays an important role in multistage carcinogenesis and involves multiple genetic alterations (31, 32). Silymarin and silibinin are reported to suppress the growth of different cancer cells (17, 33–35). Other studies with human prostate cancer cells showed that silymarin and silibinin inhibit the cell growth of androgen-dependent and androgen-independent human prostate carcinomas LNCaP and DU145 cells, respectively (15, 17). Tyagi et al. (18) showed that silymarin and silibinin induce growth inhibition and apoptotic cell death in rat prostate cancer cells. Such effects are considered to occur through perturbation of cell cycle progression, leading to G_1 arrest in a dose- and time-dependent manner, and inhibiting DNA synthesis, possibly because of an effect of G_1 arrest (17, 33, 36, 37). Cyclin D1 is involved in cell cycle during early G_1 phase (38). As the major events leading to cell proliferation occur in the G_1 phase, altered expression of cyclin D1 and their cyclin-dependent kinases might be an important step in carcinogenesis (39). Cyclin D1 overexpression was reported in human cancers (40, 41) and in murine chemically induced carcinogenesis (24, 42). Cyclin D1, which is found to be overexpressed in major of human cancers, has been regarded as a

Table 4. PCNA labeling index, cyclin D1 – positive index, and apoptotic index in the prostatic lesions

Group no.	Treatment	PCNA labeling index (%)			Cyclin D1 positive index (%)			Apoptotic index (%)		
		PIN	ADC	Nonlesional area	PIN	ADC	Nonlesional area	PIN	ADC	Nonlesional area
1	DMAB	8.8 ± 2.9* (9)	10.0 ± 2.4 (9)	4.6 ± 1.5 (5)	28.4 ± 7.2 (9)	35.7 ± 6.0 (9)	4.0 ± 1.6 (5)	1.3 ± 0.3 (9)	2.0 ± 0.5 (9)	1.2 ± 0.3 (5)
2	DMAB → 100 ppm silymarin	6.8 ± 1.7 (4)	7.4 ± 2.4 (5)	4.2 ± 2.4 (5)	25.5 ± 9.9 (4)	27.8 ± 4.4 [†] (5)	3.6 ± 0.5 (5)	1.3 ± 0.3 (4)	2.2 ± 0.4 (5)	1.2 ± 0.3 (5)
3	DMAB → 500 ppm silymarin	6.8 ± 2.2 (11)	6.3 ± 1.5 [†] (3)	4.0 ± 1.2 (5)	24.7 ± 6.3 (11)	23.0 ± 3.6 [†] (3)	3.4 ± 1.1 (5)	1.6 ± 0.3 [†] (11)	3.8 ± 0.9 [†] (3)	1.1 ± 0.5 (5)
4	500 ppm silymarin	—	—	3.4 ± 1.1 (5)	—	—	0.6 ± 0.5 (5)	—	—	1.2 ± 0.3 (5)
5	None	—	—	3.8 ± 0.8 (5)	—	—	0.6 ± 0.3 (5)	—	—	1.1 ± 0.2 (5)

NOTE: Numbers in parentheses are nos. of lesions or areas examined.

Abbreviation: ADC, adenocarcinoma.

*Mean ± SD.

[†]Significantly different from group 1 by Student's *t* test, *P* < 0.05.

[‡]Significantly different from group 1 by Student's *t* test, *P* < 0.02.

relevant molecular biomarker in cancer chemoprevention (43, 44). Silymarin and silibinin were reported to decrease in protein levels of cyclin D1 in prostate cancer cells (15, 17). In the present study, silymarin also suppressed cyclin D1 overexpression in prostate adenocarcinoma.

Also, treatment with silymarin inhibits the increase in cell proliferation activity caused by a radical-generating tumor promoter (20). Silymarin is known to exert an antipromoting effect on skin tumorigenesis in mice mediated by impairment of receptor and nonreceptor tyrosine kinase signaling pathway (19). Moreover, in an *in vivo* preclinical prostate cancer model, silibinin inhibits advanced human prostate carcinoma growth (30). In this study, the incidence of adenocarcinoma was decreased by the treatment with silymarin, whereas that of PIN in group 3 was slightly higher than group 1 without statistical significance. The reason for this is unknown, but it may be possible that silymarin feeding at a dose of 500 ppm inhibits progression of PIN to invasive adenocarcinoma. Feeding with silymarin lowered the PCNA labeling indices in the preneoplasms and/or carcinomas of prostate, suggesting that silymarin in diet could suppress the high-proliferative activity of cells initiated with a carcinogen. The other significant finding of this study is the apoptotic index of PIN and adenocarcinoma, which was found to be significantly greater in silymarin-fed rats. The results are in accordance with our previous studies (23, 25) and suggest that, in addition to inhibiting proliferation, apoptosis plays a significant role in inhibition of DMAB-induced prostate carcinogenesis by silymarin. Thus, in the current study, the inhibition of carcinogen-induced prostate malignancies for rats consuming silymarin in part is explained by the alteration of cell proliferating activity and/or apoptosis.

Chemoprevention of cancer might be defined as the deliberate introduction of these selected nontoxic substances

into the diet for the purpose of reducing cancer development. Silymarin is clinically used to as antihepatotoxic agents and devoid of any toxicity and untoward effects in both animal and human studies (45). In the present study, the estimated daily silymarin intakes in rats given diet containing 100 and 500 ppm silymarin were ~ 5 and 25 mg/kg. In a direct extrapolation to a 60 kg person, these doses are equivalent to the estimated doses of clinical use as an antihepatotoxic agent (45). Recently, Singh et al. (30, 46) reported that dietary feeding of silibinin (up to 1%) to nude mice did not show any adverse effect. In the present study, administration of 500 ppm silymarin did not also show any adverse effect on diet consumption, body weight gain, prostate weight, and pathologic alteration for 40 weeks. On the other hand, silibinin is physiologically achievable in different organs including prostate as well as in plasma, and the achievable levels of total silibinin has been found in the range of 15 to 100 μmol/L in plasma by feeding with 0.05% to 1% silibinin/silymarin in rodents (30, 47, 48). The achievable levels (15-100 μmol/L) of silibinin showed inhibition of human prostate cancer cells growth in culture (16, 17, 30). These observations showed that the efficacy of silymarin at dietary dose levels without any adverse effects could have a direct practical and translational relevance to human prostate cancer patients. However, silymarin is a mixture of three structural isomers of flavonoids. Among the flavonoids, silibinin (also called silybin, silibin, or sibilinin) is suggested to be the most active constituent. Because the cancer chemopreventive and anticarcinogenic effects of silymarin seem to be due to the main constituent silibinin (16, 30, 37, 49, 50), further studies of the chemopreventive effects of silibinin itself are necessary.

In conclusion, dietary administration of silymarin significantly suppressed the development of DMAB-induced rat prostate carcinomas. Such cancer protective effect of silymarin might relate to the modulation of cell growth and apoptosis in the prostate neoplastic lesions.

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