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Inhibitory Effects of the Natural Products Indole-3-carbinol and Sinigrin during Initiation and Promotion Phases of 4-Nitroquinoline l-Oxide-induced Rat Tongue Carcinogenesis

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The modifying effects of indole-3-carbinol (I3C) and sinigrin (SIN) on the initiation and post-initiation phases of tongue carcinogenesis induced by 4-nitroquinoline 1-oxide (4-NQO) were investigated in male ACI/N rats. Rats were divided into eight groups: group 1 was given 4-NQO (10 ppm) in the drinking watar for 12 weeks, starting at 7 weeks of age; groups 2 and 3 were given 4-NQO and fed the diets containing I3C (1,000 ppm) and SIN (1,200 ppm) for 14 weeks, respectively, starting at 6 weeks of age; groups 4 and 5 were given 4-NQO and then they were fed I3C and SIN containing diets for 23 weeks, respectively, starting one week after 4-NQO exposure; groups 6 and 7 were given I3C and SIN alone, respectively, during the experiment; group 8 served as an untreated control. At the termination of the experiment (week 37), the incidence of tongue neoplasms (squamous cell papilloma and carcinoma) in group 2 (1/15, 7%), group 3 (1/15, 7%), group 4 (3/15, 20%) or group 5 (2/15, 13%) was significantly smaller than that in group 1 (12/17, 71%) ($P=0.0003$, $P=0.005$ or $P=0.002$). No tongue carcinomas developed in rats of groups 2, 3, and 5. Similarly, the incidence of preneoplastic lesions (hyperplasia and dysplasia) of the tougue in group 2 (11/15, 73%), group 3 (10/15, 67%), group 4 (11/15, 73%) or group 5 (10/15, 67%) was significantly lower than that in group 1 (17/17, 100%) ($P=0.04$ or $P=0.02$). There were no tongue neoplasms in rats of groups 6, 7, and 8. Administration of I3C and SIN also caused significant decreases in the number and area of silver-stained nucleolar organizer regions protein (AgNORs), a new cell proliferation index, of tongue squamous epithelium. Thus, I3C and SIN inhibited rat tongue carcinogenesis in both the initiation and post-initiation phases, when administered in these respective phases together with, or following treatment with, 4-NQO.

Key words: Inhibitory effect — Indole-3-carbinol — Sinigrin — 4-Nitroquinoline 1-oxide — Rat tongue carcinogenesis

It is well known that certain chemicals or certain natural products influence the carcinogenic process, and some exert suppressing effects on chemical carcinogenesis.[1–5] A number of studies including the extensive research by Wattenberg[1] have supported the idea of "cancer chemoprevention," a term coined by Sporn and Newton.[6] Indeed, many products derived from plants have been found to inhibit chemical carcinogenesis in various organs.[4,7] However, few studies on cancer chemoprevention in the tongue have been conducted. Previously, we reported that synthetic chemicals such as butylated hydroxytoluene, butylated hydroxyanisole, disulfiram, indomethacin, and piroxicam inhibited the development of tongue neoplasms induced by 4-nitroquinoline 1-oxide (4-NQO).[8,9] Recently, an inhibitory effect of indole-3-carbinol (I3C), a major indole of cruciferous vegetables[10,11] has been reported in carcinogenesis of several organs: I3C suppresses the occurrence of forestomach, lung, breast, and liver tumors.[12–14] Sinigrin (SIN), the corresponding glucosynolate of allyl isothiocyanate[15] could decrease 7-methylguanine formation in hepatic DNA of rats given N-nitrosodimethylamine or the tobacco-specific nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK).[16,17] Our recent work demonstrated the potential inhibitory effects of these chemicals on liver carcinogenesis induced by diethylnitrosamine in rats.[18] Moreover, epidemiological data suggested that habitual intake of cruciferous vegetables containing I3C and/or SIN is related to a reduced risk of cancer development.[19–21]

Recent studies indicated that chemically induced cell proliferation might play a role in the carcinogenic process.[22] Several chemopreventive agents have been found to reduce the proliferative activity of cells exposed to carcinogens and proliferative activity is considered to be one of the biomarkers for chemoprevention studies.[23–25] We have recently demonstrated that the number of silver-stained nucleolar organizer regions (AgNORs) reflects the proliferative activity in liver, bladder and tongue carcinogenesis, and counting AgNORs dots is a useful way of obtaining data on the proliferative index of preneoplastic and neoplastic tissues.[26–28]

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In the present study, the modifying effects of the natural products I3C and SIN on tongue carcinogenesis induced by 4-NQO were examined in male ACI/N rats in order to evaluate possible application of these chemicals as chemopreventive agents in the control of human carcinogenesis. In addition, the effects of I3C and SIN on the proliferative activity of the tongue were assessed by measuring the number and area of AgNORs dots.

MATERIALS AND METHODS

Animals Male inbred ACI/N rats, which have been maintained in our laboratory, were used. At 6 weeks of age these rats were transferred to the holding room and randomized into experimental and control groups. They were housed three or four to a wire cage. The holding room was maintained at 23±2°C and 50±10% humidity, with a 12 h light/dark cycle.

Chemicals and basal diet 4-NQO was obtained from Tokyo Chemical Ind. Co., Ltd., Tokyo. I3C (>99% pure) and SIN (98% pure) were purchased from Aldrich Chemical Co., Inc., Milwaukee, WI. Powdered CE-2 (CLEA Japan Inc., Tokyo) was used as a basal diet.

Treatment of animals A total of 101 rats were divided into eight groups as shown in Fig. 1. At 7 weeks of age, groups 1–5 were given 4-NQO in the drinking water at a concentration of 10 ppm for 12 weeks. Rats in groups 2 and 3 were fed the diets containing 1,000 ppm I3C and 1,200 ppm SIN, respectively, together with 4-NQO, starting at 6 weeks of age until one week after 4-NQO exposure. Groups 4 and 5 were given 4-NQO and then one week after 4-NQO exposure, they were fed I3C- and SIN-containing diets, respectively, and were maintained on these diets for 23 weeks. Groups 6 and 7 were given I3C and SIN alone throughout the experiment. Group 8 was fed the basal diet and tap water during the experiment and served as an untreated control. The solution of 4-NQO was freshly prepared every other day: 4-NQO (1 g) was mixed with 50 ml of absolute ethanol and 4,950 ml of distilled water using a magnetic stirrer for 24 h and then diluted in tap water at a concentration of 10 ppm. Drinking water bottles were shielded from light with aluminum foil. The diets were prepared every 2 weeks and stored at 4°C until used. Stability tests of I3C and SIN in the diets were not performed during the experiment, because these chemicals are known to be quite stable. All rats were observed daily and killed at 37 weeks after the start of the experiment in order to evaluate the incidence of preneoplastic and neoplastic lesions in the oral cavity, especially the tongue. After complete necropsy of the animals, all organs were fixed in 10% buffered formalin. All tissues and gross lesions were processed for histology by conventional methods and stained with hematoxylin and eosin (H-E). Epithelial lesions (hyperplasia, dysplasia and neoplasia) in the oral cavity were diagnosed according to the criteria described by Rubio.[29]

Enumeration of AgNORs For assessment of proliferative activity of tongue squamous epithelium, the number and area of AgNORs were quantified in five animals from each group. The one-step silver colloid method for AgNOR staining[28, 30] was carried out on 3-μm paraffin-embedded sections from the animals killed at the end of the study. Computer-assisted image analysis quantitation of the number and area of AgNORs in 100 interphase cells from the nonlesional areas (lower and

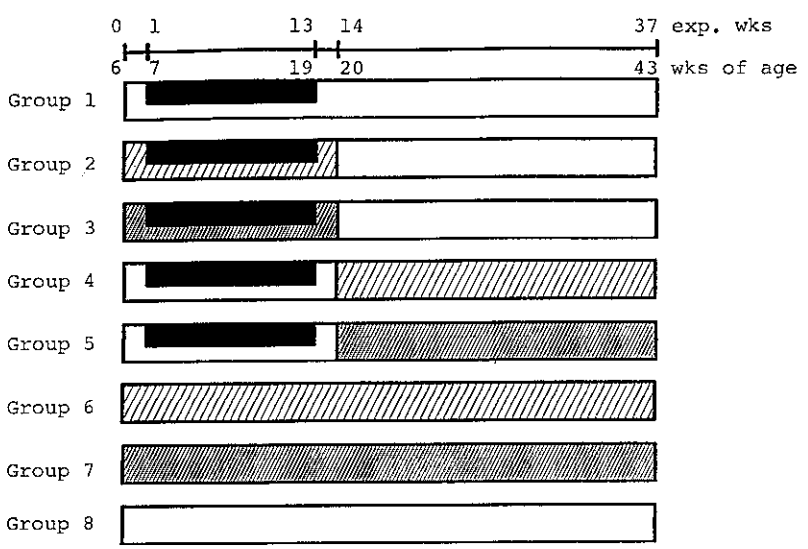

Fig. 1. Experimental protocol. ■: 4-NQO, 10 ppm in the drinking water; ▨: I3C, 1,000 ppm in the diet; ▨: SIN, 1,200 ppm in the diet; □: Basal diet, CE-2.

Table I. Body, Liver and Relative Liver Weights in Each Group

Group No.	Treatment	No. of rats examined	Body wt. (g)	Liver wt. (g)	Relative liver wt.
1	4-NQO	17	318±19[a]	10.0±0.8	3.14±0.12
2	4-NQO+I3C	15	309±34	9.9±0.7	3.22±0.19
3	4-NQO+SIN	15	310±17	10.4±1.0	3.35±0.25
4	4-NQO→I3C	15	288±37	9.2±1.5	3.18±0.21
5	4-NQO→SIN	15	283±27	8.7±1.2	3.06±0.20
6	I3C	8	302±19	9.1±0.6	3.03±0.13
7	SIN	8	308±19	9.5±0.9	3.09±0.25
8	No treatment	8	298±14	9.4±0.5	3.11±0.11

a) Mean±SD.

Table II. Incidence of Tongue Neoplasms in Rats of Each Group

Group No.	Treatment	No. of rats examined	No. of rats with tongue neoplasms (%)		
			Total	Papilloma	Carcinoma
1	4-NQO	17	12 (71)	5 (29)	12 (71)
2	4-NQO+I3C	15	1 (7)[a]	1 (7)	0 (0)[b]
3	4-NQO+SIN	15	1 (7)[a]	1 (7)	0 (0)[b]
4	4-NQO→I3C	15	3 (20)[c]	1 (7)	3 (20)[c]
5	4-NQO→SIN	15	2 (13)[d]	2 (13)	0 (0)[b]
6	I3C	8	0 (0)	0 (0)	0 (0)
7	SIN	8	0 (0)	0 (0)	0 (0)
8	No treatment	8	0 (0)	0 (0)	0 (0)

a)-*d*) Significantly different from group 1 by Fisher's exact probability test: *a*) $P=0.0003$, *b*) $P=0.00003$, *c*) $P=0.005$, *d*) $P=0.002$.

middle thirds of the epithelium in the dorsal site of the radix) was performed using an image analysis system SPICCA II (Japan Avionics Co., Tokyo) with an Olympus BH-2 microscope and a color CCD camera (Hamamatsu Photonics Co., Hamamatsu).[31]

Statistical analysis Statistical analysis of differences in the incidence of lesions or AgNOR quantitation between groups was performed by using Fisher's exact probability test or Student's *t* test.[32]

RESULTS

Rats in groups 1 through 7 tolerated well the oral administration of 4-NQO, I3C or SIN and no toxic changes were noted in the liver of rats in these groups. Body, liver, and relative liver weights of rats in all groups at the end of the study were almost the same (Table I).

Macroscopically, epithelial thickening was observed in the tongue of the rats exposed to 4-NQO. There were no gross lesions in the oral cavity of rats in groups 6–8. Papillary tumors with ulceration were observed in the tongue of rats mainly in group 1, and most were located at the dorsum.

Histologically confirmed results on tumor development are summarized in Table II. In the present study, tumor occurrence was observed in the tongue and no neoplastic or preneoplastic changes were seen in other organs. The incidence of tongue tumors in group 1 was 71% (12 of 17 rats), 7% (1 of 15 rats) in group 2, 7% (1 of 15 rats) in group 3, 20% (3 of 15 rats) in group 4 or 13% (2 of 15 rats) in group 5. These neoplasms were squamous cell papilloma (Fig. 2A) or keratinizing epidermoid carcinoma (Fig. 2B) with infiltration into the muscular layer. No metastasis was noted in rats with carcinoma. In rats of groups 2, 3, and 5, no tongue carcinomas developed. No neoplasms in any organs including the tongue were seen in rats of groups 6–8. Statistical analysis revealed that the incidences of squamous cell carcinoma of the tongue in rats of groups 2–5 were significantly smaller than that of group 1 ($P=0.00003$, $P=0.005$). Also, the incidences of total tongue neoplasms (papilloma and carcinoma) in animals of groups 2–5 were significantly lower than that of group 1 ($P=0.0003$, $P=0.005$ or

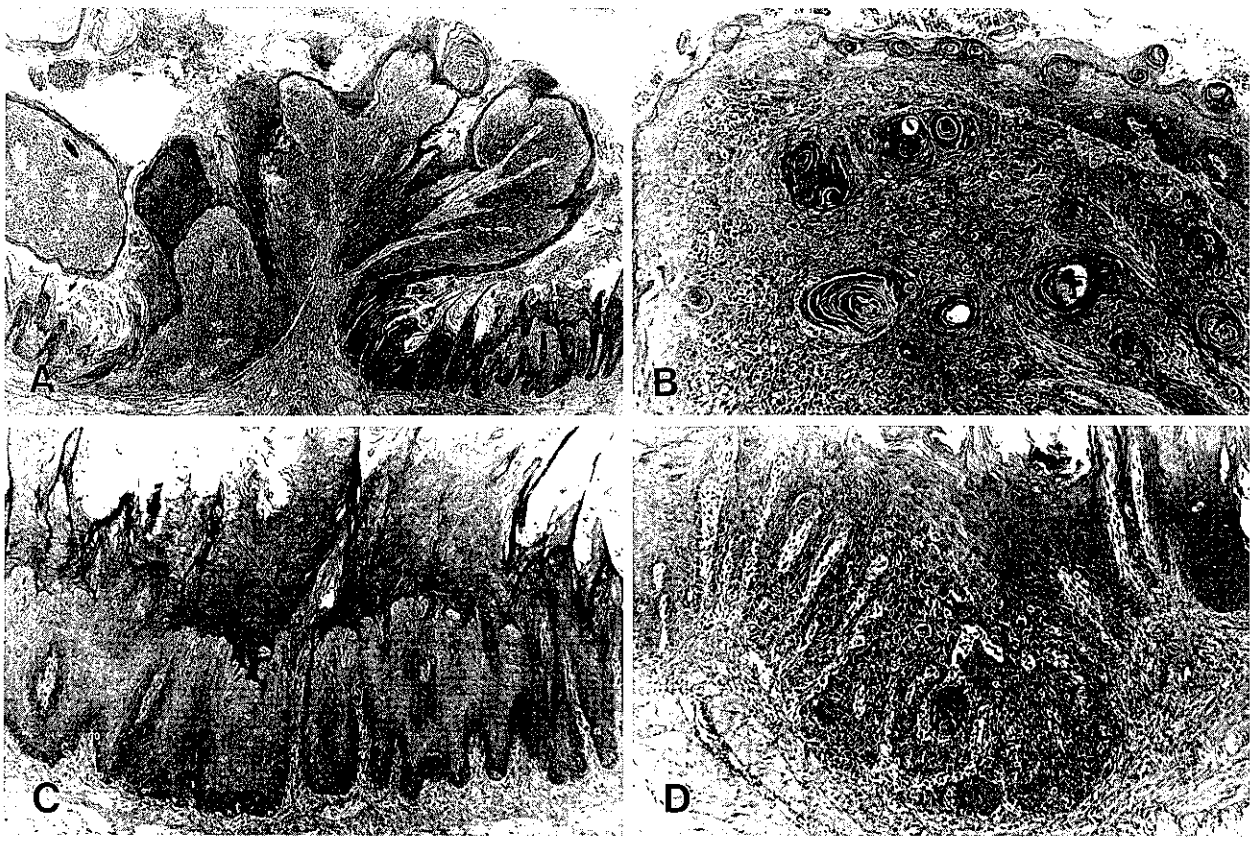

Fig. 2. (A) Squamous cell papilloma, (B) keratinizing squamous cell carcinoma, (C) squamous cell hyperplasia, and (D) squamous cell dysplasia of the tongues in different rats of group 1. H-E stain, (A) ×44, (B)–(D) ×110.

Table III. Incidence of Preneoplastic Lesions of the Tongue of Rats in Each Group

Group No.	Treatment	No. of rats examined	No. of rats with preneoplastic lesions (%)		
			Total	HP[a]	DYS[a]
1	4-NQO	17	17 (100)	12 (71)	10 (59)
2	4-NQO+I3C	15	11 (73)[b]	8 (53)	7 (47)
3	4-NQO+SIN	15	10 (67)[c]	7 (47)	5 (33)
4	4-NQO→I3C	15	11 (73)[b]	9 (60)	8 (53)
5	4-NQO→SIN	15	10 (67)[c]	10 (67)	5 (33)
6	I3C	8	0 (0)	0 (0)	0 (0)
7	SIN	8	0 (0)	0 (0)	0 (0)
8	No treatment	8	0 (0)	0 (0)	0 (0)

a) HP=squamous cell hyperplasia; DYS=squamous cell dysplasia.
b), *c*) Significantly different from group 1 by Fisher's exact probability test: *b*) $P=0.04$, *c*) $P=0.02$.

$P=0.002$). The histological examination also revealed a number of hyperplastic and dysplastic lesions (Figs. 2C and 2D) in the tongue with or without neoplasms. The incidence of these preneoplastic lesions of the tongue in each group is indicated in Table III. Such lesions were observed in rats given 4-NQO (groups 1–5). Although there were no significant differences in the incidence of hyperplastic or dysplastic lesions among the groups, the

Table IV. Number and Total Area of AgNORs of Tongue Squamous Cells in Each Group

Group No.	Treatment	No. of cells examined	No. of AgNORs per cell	Total area of AgNORs per cell (μm^2)
1	4-NQO	100	$2.36 \pm 1.03^{a)}$	3.02 ± 2.01
2	4-NQO+I3C	100	$1.77 \pm 1.76^{b)}$	$2.83 \pm 1.25^{b)}$
3	4-NQO+SIN	100	$1.85 \pm 1.81^{b)}$	$2.79 \pm 1.08^{b)}$
4	4-NQO→I3C	100	$1.76 \pm 1.68^{b)}$	$2.74 \pm 1.32^{b)}$
5	4-NQO→SIN	100	$1.79 \pm 1.55^{b)}$	$2.66 \pm 1.23^{b)}$
6	I3C	100	1.63 ± 0.56	2.56 ± 1.01
7	SIN	100	1.75 ± 0.94	2.73 ± 1.11
8	No treatment	100	1.64 ± 0.78	2.68 ± 1.02

a) Mean ± SD.
b) Significantly different from group 1 by Student's *t* test ($P<0.05$).

combined incidences of these lesions in rats of groups 2–5 were significantly lower than that of group 1 ($P=0.04$ or $P=0.02$).

The results of computer-assisted image analysis of AgNORs in the nonlesional areas of tongue in rats of each group are indicated in Table IV. Mean number and area of AgNORs in rats given 4-NQO and I3C or SIN were significantly lower than those of rats treated with 4-NQO alone ($P<0.05$). The values in rats given I3C, SIN or basal diet were almost the same.

DISCUSSION

In the present study, dietary administration of I3C and SIN during the initiation or post-initiation phase of 4-NQO-induced tongue carcinogenesis in rats clearly inhibited tumor development. Moreover, occurrence of preneoplastic lesions of the tongue epithelium was also suppressed.

Several naturally occurring substances have been reported to inhibit chemical carcinogenesis of various organs.[1–5] According to the classification described by Wattenberg,[1] they can be divided into 3 categories, namely (i) compounds that prevent the formation of carcinogens from precursor substances, (ii) blocking agents and (iii) suppressing agents. Previously, we have reported the protective effects of naturally occurring plant phenols, such as ellagic acid and chlorogenic acid on tumor development in the liver and/or colon, when they were given concurrently with a carcinogen.[33, 34] Similarly, dietary administration of I3C and SIN exerted inhibitory effects on diethylnitrosamine-induced hepatocarcinogenesis, when they were given during the initiation phase.[18] These results in the present study are similar to those of our previous studies and the two chemicals are considered to be blocking agents. In the present study, feeding of these chemicals after the carcinogen exposure also inhibited tumor development. Therefore, these chemicals possess both blocking and suppressing activity. Similar compounds having both blocking and suppressing potential, i.e. benzyl isothiocyanate and D-limonene, have been reported.[1, 2, 25] In other organs, I3C could inhibit benzo[*a*]pyrene-induced forestomach and lung neoplasms in ICR/Ha mice,[12, 13] 7,12-dimethylbenz[*a*]anthracene-induced mammary tumors in Sprague-Dawley rats[12] and aflatoxin B_1 (AFB_1)-induced hepatotumorigenesis in rainbow trout[14] when administered prior to or during carcinogen exposure. A recent study by Jang *et al.* revealed that I3C inhibited the hyperplastic nodules of the liver in a rat multi-organ carcinogenesis model.[35] However, few studies on the modifying effects of naturally occurring substances on tongue carcinogenesis in rodents have been described. In the present study, both I3C and SIN feeding during either the initiation or the post-initiation phase clearly suppressed preneoplastic and neoplastic lesions of the tongue epithelium induced by 4-NQO. On the other hand, it has been reported that post-initiation exposure of I3C enhanced AFB_1-induced liver tumorigenesis in rainbow trout[36] and I3C increased rat colonic carcinogenesis by 1,2-dimethylhydrazine (DMH) when given before, during and after DMH administration.[37] However, the doses used in the former study were higher than those consumed by humans[15] and the duration of the post-initiation phase in the latter study was too short for evaluating the efficacy of the test chemical as a chemopreventive agent. Thus, the conditions used in these two experiments are unlikely to occur in humans.

Recently, Morse *et al.* assessed the effects of SIN and I3C on DNA methylation in target tissues of NNK tumorigenesis and the effect of SIN on NNK carcinogenesis in a two-year bioassay in F344 rats.[17] They reported that dietary SIN decreased 7-methylguanine formation in hepatic DNA, but had no effect on 7-methylguanine levels of lung or nasal mucosal DNA. Dietary I3C increased 7-methylguanine levels in hepatic

DNA, but decreased DNA methylation in lung and nasal mucosa. In a bioassay, SIN had no effect on NNK tumorigenesis in these target tissues, but a greater incidence of exocrine pancreatic tumors was found in the NNK plus SIN group (6/38, 16%) than in the NNK alone group (1/40, 2.5%). They concluded that the absence of any inhibitory effect of dietary SIN on NNK liver tumorigenesis may be due to factors other than DNA methylation and commented that the contrary effects on NNK-induced hepatic DNA methylation by SIN and I3C indicate the complexity of dietary modulation of carcinogenesis. They also reported that I3C inhibits NNK-induced lung tumors in A/J mice and suggested that the basis of the inhibition is the decrease in O^6-methylguanine formation in the lung caused by I3C pretreatment.[38]

The mechanism(s) by which SIN and I3C exert their inhibitory effects on the 4-NQO-induced tongue carcinogenesis is not clear. Initially, 4-NQO was considered to react directly with DNA, RNA, and protein, but later the reaction product, 4-hydroxyaminoquinoline 1-oxide (4-HAQO), was determined to be the more likely proximate carcinogen.[39] Several substituted 4-NQO and 4-HAQO derivatives have been reported to possess carcinogenic activity in a range of animal species including rats.[40] The overall evidence suggests that the metabolic activation of 4-NQO to 4-HAQO is important.[41] The enzyme which catalyzes this reaction has been identified as DT diaphorase, a NAD(P)H-quinolineoxidoreductase.[42] In addition, these active metabolites could covalently bind to DNA bases, particularly to guanine and adenine and form DNA adducts in target tissues to produce tumors.[41, 43, 44] In an *in vitro* study, 4HAQO could cause single-strand scissions of DNA only in the presence of oxygen radical.[45] There is some evidence that indoles and glucosinolates, including I3C and SIN may alter the metabolism of carcinogens. I3C induces various mixed-function oxidase enzyme systems,[13, 46] a major detoxification enzyme, glutathione *S*-transferase (GST), and epoxide hydratase.[47–49] Similarly, cabbage and Brussels sprouts, which contain large amounts of glucosinolates and indoles enhanced GST.[47, 50] I3C decrease the covalent binding of carcinogen metabolites to DNA in mouse liver treated with benzo[*a*]pyrene or N-nitrosodimethylamine.[51] Thus, the inhibitory effects of I3C and SIN in the present study may be related to some action of these compounds on the metabolic activation, DNA adduct formation, or detoxification of 4-NQO, or radical formation. In the present study, administration of I3C and SIN caused significant decreases in the number and area of AgNORs, which are regarded as indices of cell proliferation activity.[26–28, 52] Our results also suggest that AgNOR enumeration is a useful biomarker in chemopreventive research.

In the case of post-initiation exposure to these substances, their inhibitory effects on tongue carcinogenesis may be closely related to their effects in decreasing proliferation of tongue squamous epithelium.[53] However, additional biochemical or molecular biology studies will be necessary to elucidate the exact mechanism(s).

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