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Effects of Prepubertal Indole-3-Carbinol Treatment on Development of *N*-Methyl-*N*-Nitrosourea-induced Mammary Carcinomas in Female Sprague-Dawley Rats

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Abstract. *Background: Indole-3-carbinol (I3C), which is present in cruciferous vegetables, has been shown to prevent the development of mammary cancer when administered to adult animals. However, no studies have been reported on the effects of prepubertal short-term I3C treatment on N-methyl-N-nitrosourea (MNU)-induced mammary carcinogenesis. Materials and Methods: Prepubertal female Sprague-Dawley rats were administered the vehicle (Group 1) or I3C (Group 2, 250 mg/kg/day at 15 and 16 days of age; Group 3, 50 mg/kg/day at 15 and 16 days of age; Group 4, 50 mg/kg/day at 15, 16, 29 and 30 days of age; Group 5, 50 mg/kg/day at 29 and 30 days of age). All rats were administered 50 mg/kg MNU at 22 days of age. Rats were sacrificed at 34 weeks of age or when their largest mammary tumor reached a diameter of ≥1 cm. Body weight gain, vaginal opening, estrous cyclicity and mammary carcinogenesis were compared between the groups. Results: Rats administered 250 mg/kg I3C exhibited acute toxicity, and 40% of that group died soon after administration of I3C. There was no significant difference in body weight and relative uterine-ovarian weight of surviving rats between groups at the end of the experiment. However, rats from Group 2 and Group 3 exhibited earlier vaginal opening and prolonged estrous cyclicity, respectively. I3C treatment before and after MNU administration (Group 4) tended to reduce mammary carcinoma incidence (percentage of mammary carcinomas with a diameter of ≥1 cm) and multiplicity (number of all-sized mammary carcinomas per rat), and prolonged the latency (time from MNU administration to point when mammary tumors grew to a diameter of ≥1 cm) compared with the vehicle (control) group. Mammary carcinogenesis was not altered by other I3C treatments. Conclusion: Prepubertal I3C treatment before and after carcinogen exposure appeared to provide an insignificant protection against MNU-induced mammary carcinogenesis.*

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Breast cancer is the most prominent cancer in the Western world. In Japan, breast cancer incidence and mortality are relatively low, but they are increasing. Dietary factors seem to be particularly important in the development of human breast cancer. Recent epidemiological and laboratory studies suggest that increased consumption of certain naturally occurring phytochemicals present in fruits and vegetables is directly associated with lower risk of breast cancer (1, 2). It has been reported that several compounds in fruits and vegetables possess anti-carcinogenic properties (2). One such phyto-chemical is indole-3-carbinol (I3C), a natural compound that is an autolysis product of glucosinolates and is present in cruciferous vegetables, such as cabbage, broccoli, cauliflower and Brussels sprouts (3). Chemoprevention using natural products may be an effective way to prevent breast cancer and I3C has received particularly close attention for this purpose.

In cell culture studies, I3C inhibits cell growth, arrests cell-cycle progression at the G1 checkpoint and induces apoptosis in cancer cells from several organs including breast cancer cells (4, 5). I3C exerts antiproliferative activity by regulating estradiol metabolism (6). However, I3C has been shown to suppress the growth of both estrogen-dependent and -independent breast cancer cells (4). Moreover, I3C has a stronger antiproliferative effect against human breast cancer cells than against non-tumorigenic breast epithelial cells (7). In animal studies, consumption of Brussels sprouts or I3C effectively suppressed 7,12-dimethylbenz[α]anthracene (DMBA)- and *N*-methyl-*N*-nitrosourea (MNU)-induced mammary carcinomas in rats (8-10), and suppressed development of spontaneous mammary carcinomas in C3H mice (11). Thus, I3C appears to be a potent chemopreventive agent against breast cancer.

The exact timing of initiation of human breast carcinogenesis is unclear. However, during the prepubertal period, humans are

particularly susceptible to ionizing radiation, which is the best-documented environmental breast carcinogen (12). This is consistent with the finding that the prepubertal rat mammary gland is more susceptible to the alkylating agent MNU than is the mature mammary gland (13). In rats, the prepubertal mammary gland is highly sensitive to chemopreventive agents; when chemopreventive agents are applied short-term when mammary glands are still growing, they produce permanent lifelong protection (2). Although the chemopreventive effect of I3C has been investigated using adult rodent models (8-11), no studies have been reported on the effects of short-term prepubertal I3C treatment on mammary carcinogenesis. The objective of the present study was to evaluate the effects of prepubertal short-term I3C treatment on reproductive organs and the occurrence of MNU-induced mammary carcinomas in female Sprague-Dawley rats.

Materials and Methods

Chemicals. Indole-3-carbinol (I3C) was obtained from Sigma (St. Louis, MO, USA). I3C was dissolved in a mixture of dimethylsulfoxide (DMSO) (purity ≥99%; Nacalai Tesque, Kyoto, Japan) and corn oil (Nacalai Tesque) (2:3 ratio), and was stored at 4˚C in the dark. MNU was obtained from Nacalai Tesque and was kept at –20˚C in the dark. Immediately before use, MNU was dissolved in physiological saline containing 0.05% acetic acid to a final concentration of 5.0 mg/ml, and was then shielded from light and kept on ice.

Animals. The animals used were 14-day-old female Sprague-Dawley rats (n=150; average body weight 31.9±0.5 g, 10 female pups per nursing mother) that were purchased from Charles River Japan (Hino, Japan). The rats were housed under standard laboratory conditions (22±2˚C and 60%±10% relative humidity with a 12-h light/dark cycle). I3C disrupts endocrine functions of reproductive organs in rats (14). To avoid exposure to other endocrine-disrupting chemicals, rats were housed in standard rat polyisopentene cages (TPX, Charles River Japan) with sterilized white pine chips (White Flake, Charles River Japan) as bedding. To avoid exposure to dietary phytoestrogens, rats were fed NIH-07 PLD (Oriental Yeast, Chiba, Japan) which effectively reduces adverse endocrine-disrupting activity. Water was supplied in polycarbonate bottles with rubber stoppers. Thus, xenoestrogens were eliminated from the animal environment. Throughout the experiments, the animals were cared for in accordance with the Guidelines for Animal Experimentation, Kansai Medical University.

Experimental procedures. Animals were randomly divided into 5 different treatment groups, each consisting of 30 animals (Figure 1). Because I3C is unstable, it was administered by gavage rather than by diet (10). In Group 1, vehicle (DMSO and corn oil) was administered at 15, 16, 29 and 30 days of age. In Group 2, rats were administered 250 mg/kg body weight I3C at 15 and 16 days of age. In Groups 3, 4 and 5, rats were administered 50 mg/kg I3C: Group 3, administered at 15 and 16 days of age; Group 4, administered at 15, 16, 29 and 30 days of age; Group 5, administered at 29 and 30 days of age. All rats were administered an intraperitoneal injection of 50 mg/kg body weight MNU at 22 days of age.

After weaning at 21 days of age, rats were observed daily for vaginal opening (puberty onset). Estrous cyclicity was monitored by examining vaginal smears (15) daily, at the same time each day, from 8 to 11 weeks of age. Estrous cycles were classified as follows: i) 4- or 5-day cycle (normal duration) consisting of full estrus, diestrus I and II, and proestrus period, or including an additional 24 h of diestrus (diestrus III); ii) 3-day cycle (irregular shortened); iii) 6-day cycle (irregular elongated). The length of the estrous cycle was then calculated. In each group, body weight was recorded weekly.

All animals were palpated weekly for mammary tumors and tumor location was recorded. Rats were autopsied when their largest tumor grew to a diameter of ≥1 cm, or at 34 weeks of age (31 weeks after MNU treatment). At autopsy, all visible mammary tumors were dissected, fixed in 10% neutral buffered formalin and stained with hematoxylin and eosin (HE). In addition, apparently normal mammary glands were dissected and processed to produce routine histological preparations in order to detect microscopic tumors. The histopathology of mammary tumors of all sizes was evaluated from HE-stained sections. The histological criteria used for identification of mammary tumors were based on those of a previous study (16). Mammary tumors diagnosed as adeno-carcinomas were used for analysis.

The following data were analyzed: the final body weight and uterine-ovarian weight, the number of animals with mammary carcinomas with a diameter of ≥1 cm (carcinoma incidence), the number of all-sized carcinomas per animal (carcinoma multiplicity), and latency (time from MNU administration to point when largest mammary tumor grew to a diameter of ≥1 cm).

Statistical analysis. All data were expressed as mean ± SE. Data for vaginal opening and carcinoma incidence were analyzed using the Mantel-Cox Log-rank test. Estrous cycle patterns were analyzed using the Chi-squared test. All other data were analyzed using the non-repeated measure ANOVA parametric test or the Kruskal-Wallis non-parametric test, after assurance of homogeneity of variance. For all pre-tests that produced a probability value of $p<0.05$, *post hoc* analysis was performed using Fisher's protected least significant difference test. A probability value of $p<0.05$ was considered to indicate significance.

Results

Toxicity and body weight gain. Treatment with 250 mg/kg I3C at 15 and 16 days of age (Group 2) was toxic: 40% (12/30) of rats died soon after I3C administration. In Groups 1, 3, 4 and 5, during the experiment 4, 2, 5 and 3 rats died, respectively. The dead rats were eliminated from the evaluation and the data used for analysis were obtained from the remaining rats. Although Group 5 exhibited a decrease in body weight shortly before the termination of the experiment, I3C treatment generally did not alter body weight, compared to I3C-untreated controls (Figure 2). At the end of the experiment (at 34 weeks of age), there was no significant difference in body weight or relative uterine-ovarian weight between groups (Table I).

Reproductive organ status. Rats treated with 50 mg/kg I3C at 15 and 16 days of age (Group 3) exhibited significantly earlier (39.9±0.5 days) vaginal opening than untreated controls (Group 1: 42.6±0.8 days) (Table II). Vaginal

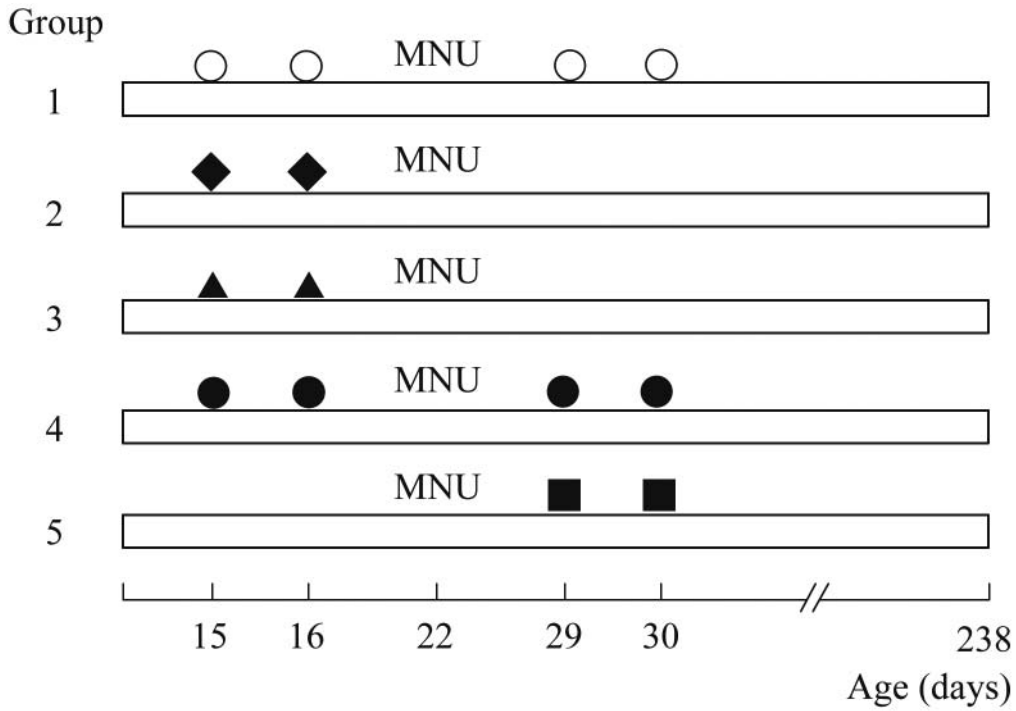

Figure 1. *Schematic representation of the experimental protocol. ○: Administration of vehicle (DMSO and corn oil) by oral gavage; ◆: administration of 250 mg/kg I3C by oral gavage; ▲, ●, and ■: administration of 50 mg/kg I3C by oral gavage. MNU: all animals were administered an intraperitoneal injection of 50 mg/kg body weight N-methyl-N-nitrosourea at 22 days of age.*

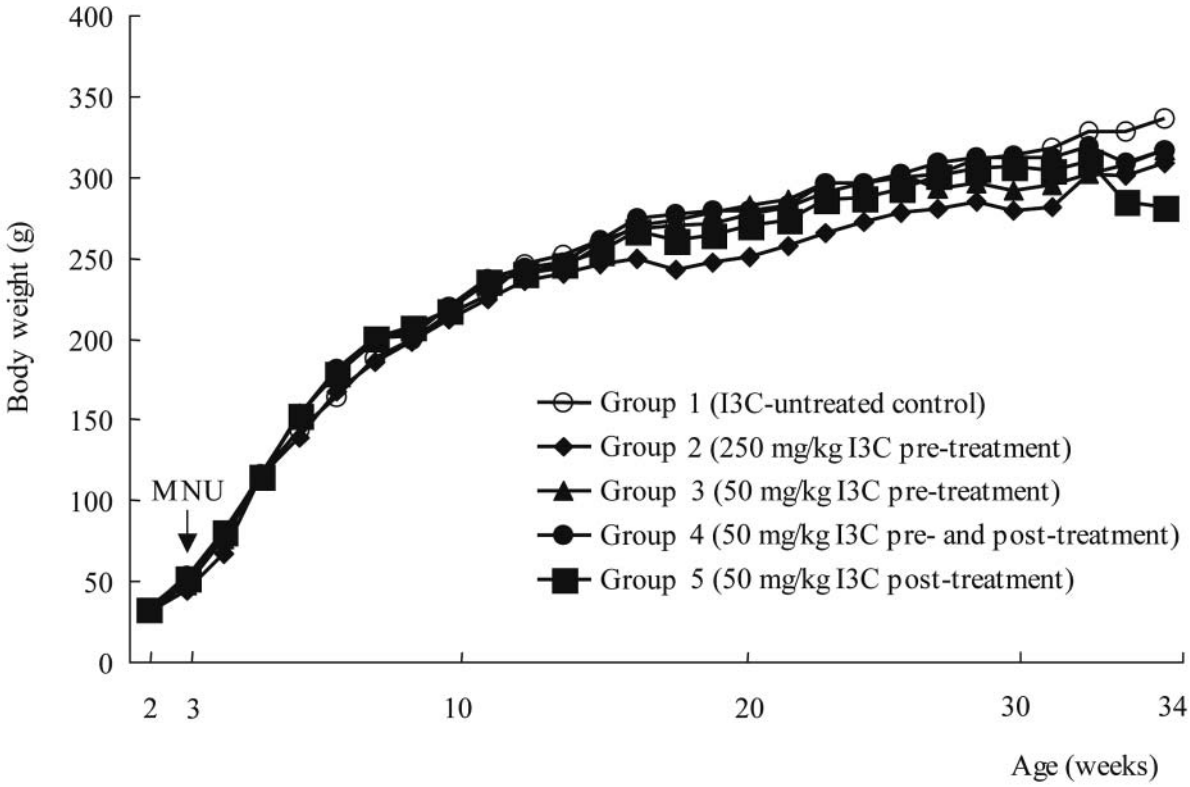

Figure 2. *Body weight in female Sprague-Dawley rats treated with indole-3-carbinol before and/or after administration of 50 mg/kg body weight MNU.*

Table I. *Final body weight and relative uterine-ovarian weight of female Sprague-Dawley rats at 34 weeks of age.*

Group	No. of rats	Body weight (g)	Relative uterine-ovarian weight (mg/100 g body weight)
1	8	336±23	350±36
2	6	309±26	281±43
3	9	317±14	324±16
4	13	316±8	319±25
5	8	281±12	318±11

Table II. *Vaginal opening and estrous cycle length of female Sprague-Dawley rats.*

Group	Vaginal opening (days)	One cycle length (days)
1	42.6±0.8	4.16±0.07
2	42.2±0.6	4.42±0.11*
3	39.9±0.5*	4.10±0.07
4	41.7±0.9	4.12±0.07
5	40.6±0.7	4.31±0.09

Values represent mean±SE; *p-value <0.05, compared with group 1.

opening was not altered in the other I3C treatment groups. Estrous cyclicity was monitored by examining rats daily, at the same time each day, from 8 to 11 weeks of age. I3C-untreated control rats (Group 1) had an average cycle length of 4.16±0.07 days. Treatment with I3C at 250 mg/kg at 15 and 16 days (Group 2) resulted in a significant increase in cycle length (4.42±0.11 days) compared with untreated controls. The cycle lengths of the other I3C-treated groups were not significantly different from that of the untreated controls.

Effects of prepubertal I3C treatments on mammary carcinogenesis. All mammary tumors with a diameter of ≥1 cm were histologically confirmed to be adenocarcinomas. I3C treatment before and after MNU exposure (Group 4) tended to delay the development of mammary carcinoma (≥1 cm), compared with I3C-untreated rats (Group 1) (Figure 3). Table III summarizes the data for mammary carcinoma incidence (percentage of rats with a mammary carcinoma with a diameter of ≥1 cm), total number of carcinomas (including mammary carcinomas with a diameter of <1 cm), multiplicity (carcinomas, of all sizes, per rat), and latency. Rats treated with I3C before and after MNU exposure (Group 4) tended to have lower mammary carcinoma incidence and multiplicity, and longer latency compared with untreated controls (Group 1), but these differences were not significant. Mammary carcinogenesis was not affected by other I3C treatments (Groups 2, 3 and 5).

Discussion

In the present study, we investigated the chemopreventive effects of short-term prepubertal administration of I3C (abundant in cruciferous vegetables) against breast cancer. It has been reported that I3C has a relatively low acute toxicity (17) and that adult rats tolerate I3C well at doses of up to 250 mg/kg (18). However, in the present study, although a 50 mg/kg dose of I3C did not cause acute toxicity in prepubertal rats, 40% (12/30) of rats administered 250 mg/kg I3C died soon after the administration. During the course of the experiment, the remaining rats administered 50 or 250 mg/kg I3C did not exhibit treatment-related toxicity, as indicated by body weight gain. In rats, I3C disrupts the functions of the female reproductive organs (14). In the present study, both 50 and 250 mg/kg I3C treatment at 15 and 16 days of age caused endocrine disruption (early vaginal opening and disturbance of the estrous cycle). However, there was no significant difference in relative uterine-ovarian weight at the end of the study (34 weeks of age) between I3C-treated and -untreated rats. There has not been extensive study of the effects of prepubertal exposure to I3C on reproductive organ development and endocrine status, as indicated by such parameters as vaginal opening and estrous cyclicity, respectively. In the present study, although prepubertal exposure to I3C did not affect the relative uterine-ovarian weight at the end of the experiment, it had an endocrine-disrupting effect on vaginal opening and estrous cyclicity. These results suggest that regulation of the mode of action of I3C in prepuberty is a complex process.

Administration of Brussels sprouts or I3C to adult rats inhibits the development of mammary cancer. Dietary administration of Brussels sprouts 2 weeks prior to and 2 weeks after a single DMBA injection has been found to suppress DMBA-induced mammary carcinomas (8). I3C administered by gavage 20 hours before a single DMBA injection or administered by diet for 8 days before a single DMBA injection has been found to inhibit DMBA-induced mammary tumor formation (9). Administration of I3C from 7 days before to 7 days after injection of DMBA or MNU has been found to suppress DMBA- and MNU-induced mammary carcinomas (10). In adult rats, Brussels sprouts and I3C have chemopreventive effects against mammary cancer when administered before or during exposure to a chemical carcinogen (in the initiation period). I3C has a greater inhibitory effect against DMBA (pro-carcinogen)-induced mammary carcinomas than against MNU (direct-acting carcinogen)-induced mammary carcinomas; it appears likely that this difference is due to the striking effects of I3C

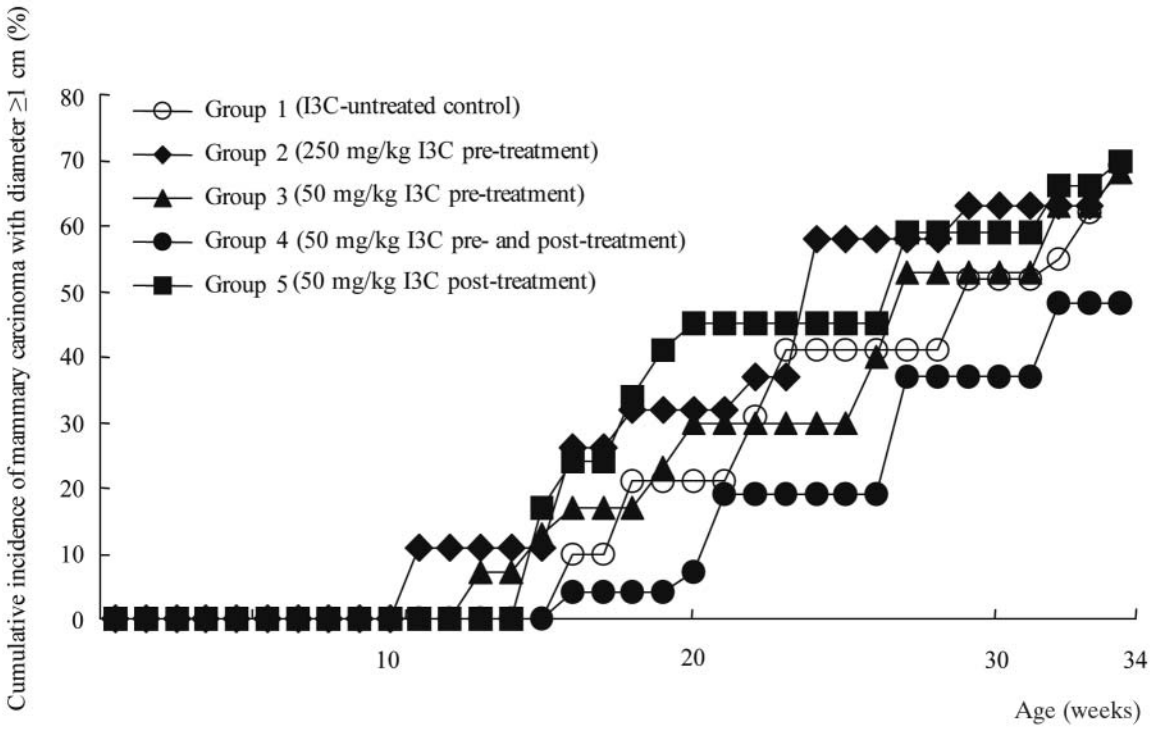

Figure 3. *Effects of prepubertal indole-3-carbinol treatment on the cumulative incidence of MNU-induced mammary tumors with a diameter of ≥1 cm in female Sprague-Dawley rats.*

Table III. *Effects of indole-3-carbinol treatment on mammary tumorigenesis in female Sprague-Dawley rats.*

Group	No. of treated rats	No. of surviving rats	No. of rats with carcinomas with diameter ≥1 cm (%)	Total no. of carcinomas	Multiplicity	Latency (days)
1	30	26	18 (69)	59	2.3±0.4	182±10
2	30	18	12 (67)	48	2.7±0.5	148±13
3	30	28	19 (68)	75	2.7±0.4	186±12
4	30	25	12 (48)	40	1.6±0.3	201±12
5	30	27	19 (70)	67	2.5±0.1	159±12

on both phase I and phase II drug-metabolizing enzymes (10). Consistent with previous reports, in the present study, I3C administered to prepubertal rats before and after MNU administration tended to suppress mammary carcinogenesis, but the effect was not significant.

Although I3C inhibits human breast cancer cell growth in culture (3, 4), administration of I3C to animals at the post-initiation phase has produced conflicting results. In some studies in which I3C was fed long-term to animals in the post-initiation phase, the incidence and multiplicity of spontaneous mammary carcinoma in C3H mice were significantly suppressed (11). However, in other such studies, only a transitory regression of DMBA-induced mammary carcinomas or an increase in latency was observed; the carcinoma incidence itself was not reduced (8, 19). Furthermore, in some studies, post-initiation administration of I3C to rats had no effect on the development of DMBA- or MNU-induced mammary carcinomas (18, 20). Thus, although studies indicate that I3C is an effective blocking agent when administered to adult animals, there is no convincing evidence that it is an effective suppressive agent. At the low pH of the stomach, I3C is metabolized to multiple products including 3,3'-diindolylmethane (DIM), and reports indicate that DIM significantly inhibits the growth of DMBA-induced mammary carcinomas in rats (21) and MCF-7 cells transplanted into athymic mice (22). In a multi-organ rat model, post-initiation

administration of I3C provided some degree of protection against mammary cancer but promoted carcinogenesis in other organs such as the liver (19) and thyroid (23).

In conclusion, in the female Sprague-Dawley rats of the present study, prepubertal short-term exposure to I3C resulted in moderate endocrine disruption and tended to protect against MNU-induced mammary carcinogenesis when administered before and after the carcinogen exposure, but the cancer-suppressive effect was not significant. The present results suggest that prepubertal administration of I3C does not provide effective chemoprevention against mammary cancer.

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