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**13. ABSTRACT (Maximum 200 Words)**
Metabolic and apoptotic genes underlying the effects of indole-3-carbinol (I3C) on breast cancer chemoprevetion were examined. A short-term (up to 10 days) treatment of rats with I3C at 5 or 25 mg/kg b.wt. ahs bee found to induce activities of caspase-3, -8, and -9 in the mammary gland, and at 250 mg/kg, upregulate the mRNA transcriptions of hepatic CYP1A1, 1B1, and 2B1/2 and mammary CYP1A1, and the oxidateive metabolism of 17b-estradiol and estrone by liver microsomes. Further, postcarcinogen treatment (thrice weekly for up to 20 weeks) of rats with tamoxifen (TAM) (10 µg/rat), I3C (250 mg/kg b.wt.) or TAM+I3C showed that the latency of malignant mammary tumors was significantly increased from ~70 to 112 days in TAM- or TAM+I3C-treated rats compared to vehicle- or I3C treated rats. The mean number of malignant mammary tumors per rat was significantly decreased in the TAM-, I3C- or TAM+I3C-treated groups, and the mean tumor mass per rat was decreased in TAM-or TAM+I3C-treated groups. The data indicate that treatment with relatively low doses of TAM effectively suppresses mammary trumorigenesis, and TAM and I3C elicit cooperative effects in suppression of mammary tumor multiplicity. The mechanisms underlying the suppressing effects of I3C may vary at different dose levels.

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## 1. OBJECTIVES

Indole-3-carbinol (**I3C**), a compound found in cruciferous vegetables, has been shown to suppress estrogen-dependent cancers, including breast cancer. This study concerns identification of metabolic and apoptotic genes underlying the mechanism of I3C action in experimental mammary carcinogenesis. Under the first aim, the effective dosage of I3C that modulates the expression of cytochrome P450s (**CYPs**) in the liver and mammary gland, and apoptotic activities in the mammary gland was determined after a short-term treatment of rats with I3C at the three dose levels. Under the second aim, the effects of long-term treatment of post carcinogen rats with I3C and/or tamoxifen (**TAM**) on mammary tumorigenesis and apoptosis will be investigated in the mammary gland and mammary tumors.

## 2. STUDY ACHIEVEMENTS AND PROJECTIONS

**Achievement 1: I3C was found to upregulate CYP expression and estrogen metabolism.** Treatments of female Sprague-Dawley rats included three dose levels of I3C, i.e. 5, 25 and 250 mg/kg b.wt., administered by oral gavage in 20% ethanol in olive oil for 4 and 10 days. Rats were sacrificed 24 hrs after the last dose, and liver and mammary glands were collected and stored at -80°C. CYP mRNA expression in the liver and mammary gland and CYP probe activities in the liver were determined. The methods included: semiquantitative reverse transcription-polymerase chain reaction (**RT-PCR**) to estimate CYP mRNA transcripts, spectrophotometric assay of P450:carbon monoxide complex, and spectrofluorimetric assays of CYP specific alkoxyresorufin *O*-dealkylase activities. The mRNA transcripts for hepatic CYP1A1, 1B1, and 2B1/2 and mammary CYP1A1 were upregulated after treatment with I3C at 250 mg/kg b.wt. This treatment also increased the capacity of liver microsomes to metabolize 17β-estradiol (**E2**) to 2-OH-E2, 2-OH-E1, 6-α-OH-E2, 6-β-OH-E2, estriol and 15-α-OH-E2, and estrone (**E1**) to 2-OH-E1, 2-OH-E1, 2-OH-E2, 6(α+β)-OH-E1 and 6α-OH-E2. *[Publication (A)]*

**Achievement 2: I3C was found to increase apoptotic activities in the mammary gland.** Apoptosis in the mammary gland after the 4- and 10-day treatments of rats with I3C at the above dose levels was examined by assays of caspase activities. Total protein from mammary gland was isolated and quantified, and the activities of caspases were measured with specific substrates (*N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide for caspase-3, *N*-acetyl-Ile-Glu-Thr-Asp-*p*-nitroanilide for caspase-8, and *N*-acetyl-Leu-Glu-His-Asp-*p*-nitroanilide for caspase-9) and the relative absorbance of the released *p*-nitroanilide at 405 nm on a plate reader. Statistical analysis was performed with ANOVA. After a 4-day treatment at 5 mg/kg b.wt., I3C significantly ($P < 0.05$) increased the activities of caspase-3, caspase-8 and capsase-9 (3.3-, 2.6- and 2.5-fold, respectively) *(Table 1, **Appendix 1**)*. After a 10-day treatment at 25 mg/kg b.wt., the caspase activities were increased by 3.6-, 1.8-, and 2-fold, respectively. The dose-related effects of I3C on caspase activities in mammary gland were further confirmed using Western blot analysis to measure a 85 kD fragment from poly (ADP-ribose) polymerase (**PARP**), an apoptotic marker that is cleaved by activated caspase-3 in the early stage of apoptosis. In addition, the effects of 3,3'-diindolylmethane (**DIM**), a major condensation product of I3C in vivo, at dose levels of 8.4 and 42 mg/kg b.wt., i.e., equimolar to those of 5 and 25 mg/kg b.wt. of I3C, respectively, were also examined in the mammary gland. No

significant induction of caspase-3, caspase-8 or caspase-9 activity was observed ($P > 0.05$) *(Table 1, **Appendix 1**)*, indicating that I3C-induced apoptotic activities in the mammary gland are not due to DIM. *[Publication (B), **Appendix 2**]*

**Achievement 3: post-initiation treatment with TAM, I3C or TAM+I3C suppresses mammary gland tumorigenesis.** Two weeks after an oral dose of 7,12-dimethylbenz[*a*]anthracene (**DMBA**) at 65 mg/kg b.wt. or vehicle olive oil, rats were treated 3 times per week for up to 20 weeks with TAM (10 μg/rat), I3C (250 mg/kg b.wt.), TAM+I3C or their respective vehicles. At selected time intervals during treatment, blood samples were collected and rats were palpated and recorded for tumors. Histologic examination of mammary gland and tumors revealed that the majority of tumors were mammary adenocarcinomas. The latency of malignant mammary tumors was significantly increased in groups treated with TAM (119 days) and TAM+I3C (112 days) compared to the group treated with I3C (70 days) or with vehicle (72 days). At 112 days after DMBA, the estimated malignant tumor incidence was lower in the group treated with TAM (46.0±12.9%), I3C (64.9±11.9%) or TAM+I3C (50.7±12.1%) than that in vehicle-treated group (83.9±8.49%). The mean number of malignant mammary tumors per rat significantly decreased in the group treated with TAM (60%), I3C (30%) or TAM+I3C (71%) compared to that of vehicle-treated group. TAM- or TAM+I3C-treated group had significantly decreased mean tumor weight per rat compared to vehicle-treated group. Although the latter effect was due to TAM, the overall results suggest that TAM and I3C elicit cooperative effects in mammary tumor suppression. *[Data were reported at a poster session, the Second Annual AACR International Conference on Frontiers in Cancer Prevention Research, October 2003 (**Appendix 3**)]*

**Projection 1: determination of the effects of I3C *in vivo* on the levels of CYPs responsible for estrogen synthesis in rat ovaries and mammary gland.** Treatment of rats with I3C may have effects not only on the metabolism of estrogens, but also on the biosynthesis of estrogens. We described (under Achievement 1) that the hepatic and mammary gland CYP1A1 and hepatic CYP1B1 and 2B1/2 involved in the metabolism of estrogens were increased by I3C treatment, and so were the rates of oxidative metabolism of E1 and E2. However, the effect of I3C on the activities of CYPs catalyzing the biosynthesis of estrogens, such as CYP17 and CYP19, remains unclear. Depending on the substrate, CYP19 converts androstenedione and testosterone to E1 and E2, respectively. It is therefore necessary to measure the levels and activities of CYP17 and 19 in I3C-treated rats. The expression level will be assayed by RT-PCR as described by us previously *[Publication (A)]*. Total protein from mammary gland, mammary tumors and ovaries will be isolated and quantified, and CYP17 lysae activity will be measured with the substrate [21-$^3$H]17α-hydroxypregnenolone (1) and CYP19 activity with androst-4-ene-3,17β-dione,[1-$^3$H(N)] *(2, 3)*.

**Projection 2: determination of apoptotic activities in the mammary gland of TAM-, I3C- and TAM+I3C-treated rats and in mammary tumors of DMBA-induced and TAM-, I3C- and TAM+I3C-treated rats.** The two compounds TAM and I3C showed the variable inhibitory effects on the latency, incidence and multiplicity of DMBA-induced mammary adenocarcinomas (described under Achievement 3), but the mechanisms remain unclear. Our previous data *(Table 1, **Appendix 1**)* suggested the pro-apoptotic effects of I3C in the rat mammary gland through increased caspase activities. It is therefore hypothesized

that I3C and/or TAM may also use the same strategy to suppress mammary tumorigenesis in DMBA-induced rats. The activities of caspase-3, -8 an -9 in the mammary gland of TAM-, I3C- and TAM+I3C-treated rats and in the DMBA-induced mammary tumors of TAM-, I3C- and TAM+I3C-treated rats will be measured by the colorimetric assay described by us *[Publication (B), **Appendix 2**]*. In addition, the extent of apoptosis in the mammary gland and tumors will be also determined by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (**TUNEL**) assay *[Publication (B), **Appendix 2**]*.

**Projection 3: improvement and modification of measurements of apoptotic activities.** As initiators, caspase-8 and -9 are activated during the upstream of apoptosis cascade *(4)*. They then activate the effector caspases including caspase-3, -6 and -7, which in turn dismantle intracellular structures and result in the apoptotic phenotype. Although the activities of caspase-8 and -9 reflect the apoptotic process, the total activity of the three effectors caspase-3, -6 and -7 would be more accurate for this purpose. Since caspase-7 as well as caspase-3 cleaves *N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide, the caspase-3 assay data actually reflect the total activity of caspase-3 and -7. Based on the activities of only caspase-3 and -7, however, it may be insufficient to conclude whether it is through apoptosis that I3C suppresses mammary tumorigenesis. Caspase-6 is, therefore, highly recommended for inclusion in the assays. Knowledge of the effector caspase activities in the mammary gland and mammary tumors through assay of caspase-8 and -9, as well as caspase-3 and -6 activities may help to elucidate the mechanism underlying the tumor-suppressing effects of I3C.

The incubation time for different caspase assay will be optimized. Individual caspases have different activity level in the same tissue and may be inactivated at different rates during incubation. Our recent pilot study revealed differences in specific activities of the individual caspases with different incubation time. This is contradictory to the information provided by commercial supplier. It is therefore necessary to individualize the incubation time for each caspase to obtain the correct and accurate activity data. A time-course detection of the absorbance at 405 nm of the *p*-nitroanilide released from the respective substrates will be carried out for this purpose.

It is also proposed to measure cytochrome *c* released from mitochondria. The release of cytochrome *c* is triggered by the translocation of Bax and furthers the cascade of apoptosis. Compared to activation of initiator caspases, the release of cytochrome *c* is a much earlier event in the cascade. Cytochrome *c* appears at an elevated level in the cytosol shortly after the cells undergo apoptosis, but not necrosis. Without damaging mitochondria, cytosolic and mitochondrial fractions will be separated and relative concentrations of cytochrome *c* in each fraction will be measured by Cytochrome *c* Oxidase Kit (Sigma).

## 3. PUBLICATIONS

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liver by treatment of rats with Inodle-3-carbinol. Biochem. Pharmacol. 2002, 64: 393-404.

B. Zhang, X. and Malejka-Giganti, D. Effects of treatment of rats with indole-3-carbinol on apoptosis in the mammary gland and mammary adenocarcinomas. Anticancer Res. 2003, 23: 2473-9. **(Appendix 2)**

C. Malejka-Giganti, D., Niehans, G.A., Bennett, K.K., Parkin, D.R., Decker, R.W., and Bliss, R.L. Suppression of mammary gland carcinogenesis by post-initiation treatment of rats with tamoxifen or indole-3-carbinol or their combination. Frontiers in Cancer Prevention Research, the Second Annual AACR International Conference, October 26-30, 2003, Abstract C158, page 91-92. **(Appendix 3)**

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## Appendix 1

Table 1. Effect of a 4-day treatment of rats with I3C or DIM on caspase activities in the mammary gland (mean±SD).

| Compound | Dose (mg/kg b.wt.) | Caspase-3 | Caspase-8 | Caspase-9 |
|---|---|---|---|---|
| I3C | 0 [a] | 0.063±0.006 | 0.046±0.011 | 0.037±0.010 |
| | 5 | 0.211±0.054[b] | 0.119±0.038[c] | 0.091±0.010[c] |
| | 25 | 0.105±0.025 | 0.081±0.034 | 0.073±0.051 |
| | 250 | 0.123±0.046[c] | 0.103±0.052 | 0.081±0.039 |
| DIM | 0 [a] | 0.101±0.038 | 0.036±0.013 | 0.016±0.006 |
| | 8.4 | 0.132±0.047 | 0.040±0.008 | 0.017±0.002 |
| | 42 | 0.152±0.075 | 0.041±0.005 | 0.018±0.009 |

[a]Vehicle-treated rats.

[b]Statistically significant ($P<0.01$) compared to vehicle-treated group.

[c]Statistically significant ($P<0.05$) compared to vehicle-treated group.

# Appendix 2

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# Effects of Treatment of Rats with Indole-3-Carbinol on Apoptosis in the Mammary Gland and Mammary Adenocarcinomas

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**Abstract.** *Induction of apoptosis is an approach to suppress carcinogenesis. The effects of a 12-week treatment of female Sprague-Dawley rats with indole-3-carbinol (I3C), β-naphthoflavone or vehicle (40% ethanol in corn oil), by oral gavages starting 3 weeks after initiation of mammary tumorigenesis with 7,12-dimethylbenz[α]anthracene, on apoptotic activities in the mammary adenocarcinomas were examined. Apoptotic cells in tumor sections were detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling and quantitated by light microscopy and an Image-Plus Program. Activities of caspase-3, caspase-8 and caspase-9 were determined by colorimetric assays using the specific substrate and total tumor protein. There were no significant treatment-related effects on the numbers of apoptotic cells and caspase activities in the mammary adenocarcinomas. Likewise, protein expression levels of Bcl-2 and Bax genes in these tumors, determined by Western blot analysis, showed no treatment-related stimulation of apoptotic process. In the absence of tumorigenesis, the activities of caspase-3, caspase-8 and caspase-9 were increased up to ~3.6-fold in the mammary gland of rats treated with I3C at 5 or 25 mg/kg of body weight for 4 or 10 days. The I3C-effected induction of caspase-3 activity in the mammary gland was further confirmed by the cleavage of poly (ADP-ribose) polymerase. Treatment of rats with 3,3'-diindolylmethane, a major product of I3C in vivo, at the dose levels equimolar to those of I3C above, did not increase the caspase activities in the mammary gland. Thus, this I3C dimer does not seem to account for the increases of apoptotic activities in the mammary gland observed with I3C. The results suggest that increase of apoptosis in the mammary gland induced by I3C before initiation of tumorigenesis may contribute to suppression of tumor development.*

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*Key Words:* Indole-3-carbinol, apoptosis, mammary gland, mammary adenocarcinoma.

Indole-3-carbinol (I3C), a natural compound from the hydrolysis of glucobrassicin in cruciferous vegetables (*e.g.* cabbages, broccoli and brussels sprouts), and β-naphthoflavone (β-NF), a synthetic flavone, administered before or during exposure to carcinogen, inhibit carcinogenesis in laboratory rodents including mammary gland tumors in the rat (1,2). This effect is ascribed to I3C- or β-NF-induced increases in the levels of expression and activities of phase I and phase II enzymes resulting in increased rates of the detoxification of carcinogen. Induction of several of these enzymes by β-NF or I3C is controlled by the aryl hydrocarbon receptor (AhR), a ligand-dependent transcription factor (3-5). After treatment of medaka embryos (6), juvenile catfish (7) and the dorsal midbrain of zebrafish embryos (8) with the AhR agonists including β-NF, stimulation of the AhR and induction of a specific cytochrome P450 (CYP1A1) paralleled increases in apoptosis.

I3C has been shown to induce $G_1$ cell cycle arrest, cell growth inhibition and apoptosis in human breast and prostate cancer cells *in vitro* (9-15). In MCF-7 and MDA-MB-23 breast cancer cells (estrogen receptor-proficient and -deficient cell lines, respectively), I3C activated an antiproliferative pathway *via* decrease in the expression of cyclin-dependent kinase 6 (CDK6) by targeting a specific site in the CDK6 promoter (11) and an increase in the levels of the p21 and p27 CDK inhibitors (10). In MDA-MB-435 breast cancer cells, I3C altered the ratio of Bax:Bcl-2 and translocation of Bax to mitochondria to induce apoptosis (13). In PC-3 prostate cancer cells, I3C inhibited the phosphorylation and subsequent activation of Akt kinase and Bcl-$x_L$, and decreased expression of BAD proteins (14). Furthermore, 3,3'-diindolylmethane (DIM), an acid condensation product of I3C, formed both *in vivo* and *in vitro* (3,4), induced apoptosis in several human breast cancer cell lines independent of p53 pathway (16), and also independent of estrogen receptor status by a process that is mediated through the expression of apoptotic regulatory factors of the Bcl-2 family (17). Treatment of breast cancer cells with DIM also led to an increased expression of the p21 CDK inhibitor, resulting in a $G_1$ cell cycle

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arrest (18). Hence, activation of apoptotic process by I3C or its product(s) formed *in vivo* might lead to suppression of tumorigenesis.

Treatment of mammary tumor-bearing rats with DIM (10 oral doses at 5 mg/kg body weight (b.w.) within 20 days), supressed the tumor growth (19). In a multi-organ carcinogenesis initiated with three carcinogens, treatment of rats with I3C transiently delayed mammary tumor latency (20). In our study, rats treated with I3C starting 3 weeks after initiation of mammary carcinogenesis with 7,12-dimethylbenz[α]anthracene (DMBA) showed a trend to develop fewer mammary adenocarcinomas, with a greater average weight per tumor per rat than rats treated with β-NF or vehicle (21). The above results suggested that I3C or its product(s) might affect mammary tumor development and growth possibly *via* modulations of apoptotic activities in the tumors or tumor target tissue.

The goals of the present study were to examine: 1) the effects of a long-term (up to 12 weeks) post-initiation treatment of rats with I3C or β-NF on the levels of apoptotic activities in the DMBA-induced mammary adenocarcinomas, and 2) the effects of a short-term (4 to 10 days) treatment of rats with I3C or its major *in vivo* product DIM on apoptosis in the normal mammary gland.

## Materials and Methods

*Rat tissues.* Mammary adenocarcinomas were from rats used in our earlier study (21). Briefly, female Sprague-Dawley rats (Specific Pathogen Free) from Harlan Sprague Dawley (Indianapolis, IN, USA) were maintained on Teklad certified rodent diet from Harlan Teklad (Madison, WI, USA). Diet and water were *ad libitum*. Tumorigenesis was initiated in 7-week-old rats with one oral dose of DMBA (20 mg per rat). Three weeks after initiation, rats started treatment with I3C (250 mg/kg (b.w.) or β-NF (20 mg/kg b.w.) or vehicle (40% ethanol in corn oil) (2.5 ml/kg b.w.) by oral gavage three times per week for up to 12 weeks. Mammary tumors were removed and the tumor portions were fixed in buffered formalin, embedded in paraffin, sectioned, stained and examined microscopically. The remaining tumor tissue was frozen in liquid nitrogen and stored at -70°C. Mammary gland was from rats treated by oral gavage with I3C (5, 25 or 250 mg/kg b.w.) or vehicle (20% ethanol in olive oil) once daily for 4 or 10 days (22) or with DIM (8.4 or 42 mg/kg b.w.) for 4 days in the same vehicle. The mammary tissue frozen in liquid nitrogen and stored at -70°C was used herein.

*Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay.* Paraffin-embedded mammary tumor sections were deparaffinized with xylene (3 times 5 minutes each) and rehydrated with ethanol and water (absolute, 95%, 90%, 80%, 70%, and water 5 minutes each). After incubating with proteinase K (Sigma Chemical Co., St. Louis, MO, USA) (20 μg/ml) for 1 hour at 37°C, and a blocking solution (0.3% $H_2O_2$ in methanol) for 10 minutes at room temperature, tissue sections were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 minutes on ice and then incubated with 50 μl of TUNEL reaction mixture (Roche, Indianapolis, IN, USA) in a humidified chamber for 1 hour at 37°C. Converter-peroxidase (50 μl) was added to each section and the slides were incubated for 30 minutes at 37°C in a humidified chamber followed by incubation with 50 to 100 μl of the 3,3'-diaminobenzidine substrate solution for 10 minutes at room temperature. After rinsing with a 1x phosphate-buffer saline, the slides were covered with glass coverslips using mount medium and analyzed under light microscopy with an Image-Plus Program. The number of apoptotic cells and total cells in a selected field were recorded and expressed as the percentage of apoptotic cells. For each tumor section, 6 to 8 fields were selected and counted.

*Caspase activity assay.* Mammary tumor or mammary gland (100 mg to 200 mg tissue) frozen in liquid nitrogen was powdered in a Bessman pulverizer, and homogenized (glass/glass homogenizer) with 3 parts (w/v) of cell lysis buffer (Promega, Madison, WI, USA) on ice. The tissue lysate was centrifuged at 15,000 x g for 20 minutes at 4°C. The supernatant was collected and the protein concentration determined with Coomassie Plus Protein Reagent (Pierce, Rockford, IL, USA). The *p*-nitroaniline (pNA)-conjugated specific substrate stock solution and the caspase assay buffer (25 mM HEPES, 0.1% CHAPS, 5% sucrose, 5 mM dithiothreitol, 2 mM EDTA, pH 7.5) were mixed at room temperature before use. The acetyl-Asp-Glu-Val-Asp-pNA (Ac-DEVD-pNA), acetyl-Ile-Glu-Thr-Asp-pNA (Ac-IETD-pNA) or acetyl-Leu-Glu-His-Asp-pNA (Ac-LEHD-pNA) (Sigma Chemical Co) (0.2 mM) for caspase-3, caspase-8 or caspase-9, respectively, was placed into the wells followed by adding tissue protein (75-100 μg). Controls consisted of protein inactivated by immersion of tubes in boiling water for 5 minutes. The plate was incubated at 37°C for 4 hours or overnight at room temperature and absorbance at 405 nm determined on a plate reader (Packard BioScience, Downers Grove, IL, USA). The assays were carried out in triplicates for each tumor or mammary gland and the average absorbance at 405 nm was calculated.

*Western blot analysis.* Total protein from tumor or mammary gland lysate was used for the analysis on a precasted NuPAGE Bis-Tris gel (Invitrogen, Carlsbad, CA, USA). Electro-transfer of proteins was performed using Hoefer TE 70 Series Semi-Dry transfer unit (Amersham, Piscataway, NJ, USA) in 1 hour. The membrane with proteins was incubated in a blocking solution (5% milk in 1x Tris-buffered saline-Tween 20 (TBS-T) for at least 1 hour or overnight at 4°C, and then incubated with the primary antibody in the above blocking solution for 1 hour at room temperature with shaking. After washing with 1x TBS-T, the membrane was incubated with the secondary antibody in the same blocking solution for 1 hour with shaking at room temperature. The membrane was developed with ECL Detection Kit (Amersham) for 1 to 5 minutes and then exposed to Hyper film for a short time.

*Statistical analysis.* All data are expressed as mean±standard deviation (SD), and statistical analysis was performed using a one-way analysis of variance (ANOVA), followed by a Student's *t*-test. Differences at $p \leq 0.05$ were considered significant. In addition, the Kruskal-Wallis nonparametric eqivalent of ANOVA was used for small sample sizes or data that is not normally distributed.

## Results

*Apoptosis in mammary adenocarcinomas of I3C-, β-NF- or vehicle-treated rats.* The presence of apoptotic cells in the mammary adenocarcinomas from rats treated with I3C, β-NF

or vehicle (40% ethanol in corn oil), for up to 12 weeks starting 3 weeks after initiation of tumorigenesis with DMBA (21), was assessed by TUNEL assay. Apoptotic cells were detected in all tumors examined and constituted 4.93±3.31% (n=12), 3.61±1.72% (n=11) and 2.95±1.23% (n=10) of total cells in tumors of I3C-, β-NF- and vehicle-treated rats, respectively (Figure 1). Although the numbers of apoptotic cells in mammary adenocarcinomas of I3C- and β-NF-treated rats were somewhat greater (1.6- and 1.2-fold, respectively) than in tumors of vehicle-treated rats, no statistically significant differences were found among these groups ($p$=0.088).

Activation of proteolytic cysteine proteases called caspases is a key step in the apoptotic program (23). The activities of caspases were determined by a colorimetric assay to measure the release of pNA from the specific tetrapeptide substrates for caspases. The relative absorbances for caspase-3 activity were 0.321±0.158 (n=20), 0.372±0.136 (n=15) and 0.306±0.154 (n=16) for tumors of I3C-, β-NF- and vehicle-treated rats, respectively (Figure 2). The values for caspase-3 activity were not significantly different among the treatment groups ($p$=0.11). Likewise, the relative absorbances for caspase-8 activity: 0.178±0.090 (n=12), 0.225±0.075 (n=12) or 0.225±0.083 (n=12) for tumors of I3C-, β-NF- or vehicle-treated rats, respectively, and caspase-9 activity: 0.175±0.075 (n=8), 0.243±0.105 (n=8) or 0.152±0.100 (n=10) for tumors of I3C-, β-NF- or vehicle-treated rats, respectively, were not significantly different among these groups ($p$>0.05). Thus, post-initiation treatment of rats with I3C or β-NF did not alter the level of apoptotic activities intrinsic to mammary adenocarcinomas at the stage of development they were examined. The majority of mammary adenocarcinomas in this study were from rats that completed 12 weeks of treatment with 36 doses of I3C, β-NF or vehicle. A few tumors in each group were from rats terminated between 6 and 12 weeks of treatment. The latency of mammary adenocarcinomas was 8 to 25 weeks and the individual tumor weight varied from 0.23 to 4.8 g. There was no correlation between caspase-3 activity and tumor latency, tumor mass or duration of treatment (evaluated statistically by Pearson's correlations).

In the control of apoptotic pathway, both Bax gene that promotes cell death and Bcl-2 that is an anti-apoptotic gene have been implicated to play an important role (13, 23). Hence, the levels of Bax and Bcl-2 gene expressions were determined by Western blot analysis to assess whether treatment of rats with I3C or β-NF affects these genes in the mammary adenocarcinomas. The level of Bax gene expression did not seem to be altered by I3C or β-NF compared to the vehicle treatment, and that of Bcl-2 gene appeared to be slightly increased in the tumors of I3C- or β-NF-treated rats (Figure 3). The results suggest that treatment with I3C or β-NF does not up-regulate the apoptotic program in the mammary adenocarcinomas.

*Apoptotic activities in the mammary gland of I3C- or DIM-treated rats.* The effects of 4- and 10-day treatment of rats with I3C at 5, 25 and 250 mg/kg b.w. or vehicle (20% ethanol in olive oil) on caspase-3, caspase-8 and caspase-9 activities in the mammary gland were examined. After 4-day treatment, I3C at 5 mg/kg b.w. significantly increased the activities of caspase-3, caspase-8 and caspase-9 (3.3-, 2.6- and 2.5-fold, respectively), and I3C at 250 mg/kg b.w. increased ~2-fold caspase-3 and caspase-8 activities in the mammary gland compared to control rats (Table I). After 10-day treatment with I3C at 5 mg/kg b.w., caspase-3 activity remained increased (2.4-fold), and after 25 mg/kg b.w., the activities of caspase-3, caspase-8 and caspase-9 were also significantly increased (3.6-, 1.8- and 2-fold, respectively). The data show that there was no linear association between the extents of I3C-dependent increases in caspase activities and the dose of I3C administered or duration of treatment.

To confirm the I3C-dependent increases of caspase activities at a molecular level, stimulation of downstream events of caspase cascade-mediated apoptosis, such as the cleavage of poly (ADP-ribose) polymerase (PARP) resulting from activation of caspase-3, was examined in the mammary gland of I3C-treated rats by Western blot analysis. The cleavage of PARP was shown by the presence of an 85kD band (Figure 4). Consistent with increases of caspase-3 (Table I), a PARP-cleaved fragment (85 kD) was increased by the 4-day treatment with I3C.

Since DIM appears to be a major transformation product of I3C *in vivo* (24), the effects of a 4-day treatment of rats with DIM at the dose levels of 8.4 and 42 mg/kg b.w., *i.e.* equimolar to those of 5 and 25 mg/kg b.w. of I3C, respectively, on caspase activities in the mammary gland were examined (Table II). Slight increases in caspase-3 activity of DIM-treated rats were not significant, and likewise, there were no effects of DIM on caspase-8 and caspase-9 activities ($p$>0.05). The data suggest that I3C-induced apoptotic activities in the mammary gland are not due to DIM.

## Discussion

Apoptosis, the programmed cell death, is a complex process (23). The extent of apoptosis may be assessed by immunocytochemical (*e.g.* TUNEL assay), biochemical (*e.g.* activities of caspases) and molecular (*e.g.* expression of the Bcl-2 family genes) assays. The process and mechanisms of apoptosis have been chiefly investigated in cell systems *in vitro*. Exposure of human breast and prostate cancer cells in culture to I3C affects several molecular and signaling pathways that lead to G1 cell cycle arrest, inhibition of cell proliferation and apoptosis (9-15). To our knowledge, no attempt has been made to date to evaluate the effects of I3C on apoptosis *in vivo*. In the present study, the apoptotic activities in DMBA-induced mammary adenocarcinomas

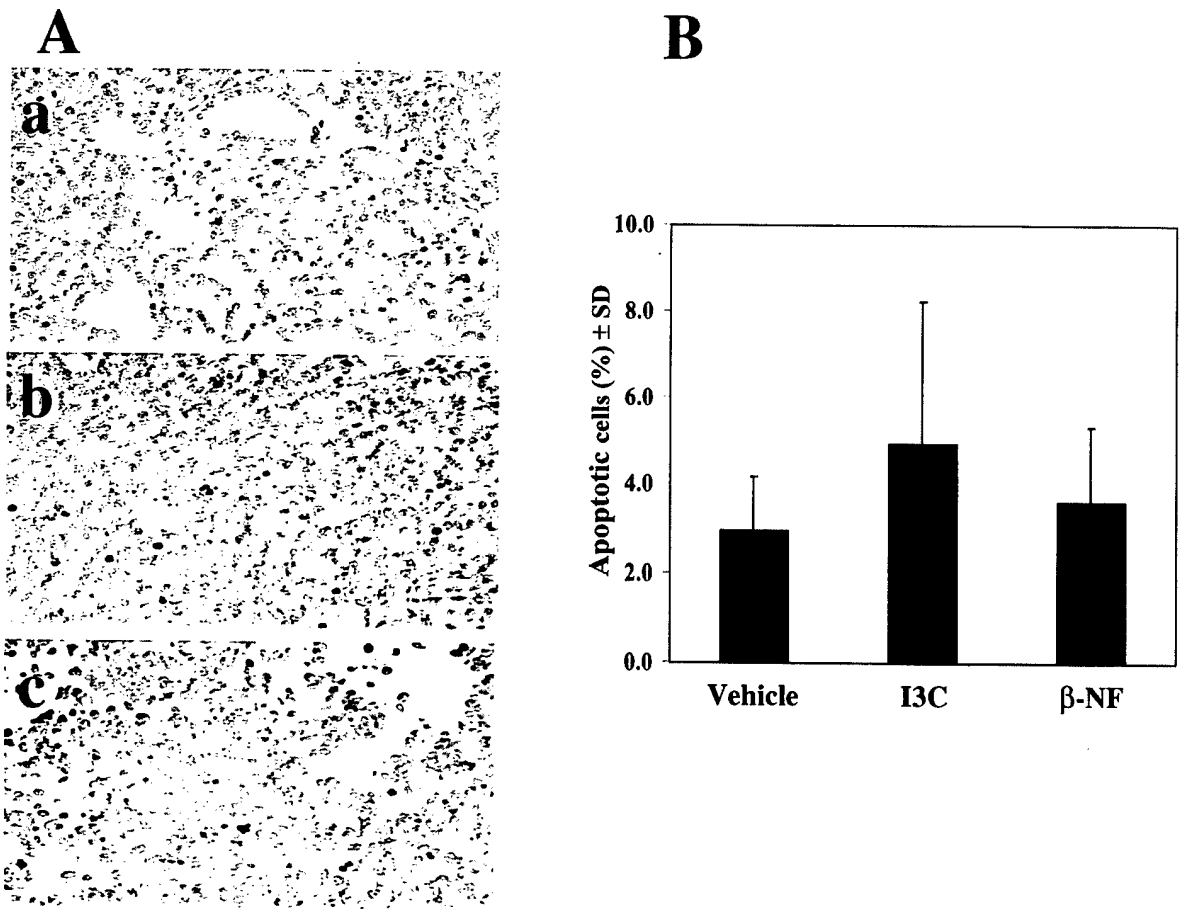


Figure 1. *Apoptotic cells in mammary adenocarcinomas determined by TUNEL assay. Stained sections were analyzed under light microscopy with Image-Plus Program. (A). Representative sections of mammary adenocarcinomas from: a. Vehicle-; b. I3C-; or c. β-NF-treated rats. (Note, dark-stained apoptotic cells). (B). Apoptotic cells (%) are expressed as the number of apoptotic cells per total number of cells in a selected field. For each tumor section, 6 to 8 fields were counted. The values are the mean±SD from 10 to 12 tumors in each treatment group.*

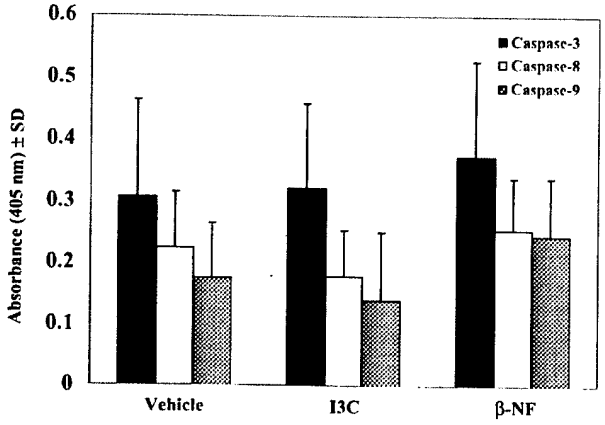


Figure 2. *Caspase activities in mammary adenocarcinomas of vehicle-, I3C- or β-NF-treated rats. The activities of caspases are expressed as the absorbance at 405 nm after adjustment for that of controls. The data are the mean±SD from 12 to 20 tumors for each treatment group. Triplicate determinations were performed for each tumor.*

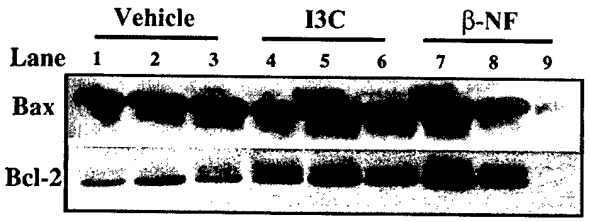


Figure 3. *Western blot analysis of Bax and Bcl-2 gene expression in mammary adenocarcinomas of vehicle-, I3C- or β-NF-treated rats. Tumors from 3 rats of each treatment group: vehicle (lanes 1-3); I3C (lanes 4-6); and β-NF (lanes 7-9), were analyzed.*

Table I. *Effect of treatment of rats with I3C on caspase activities in the mammary gland.*

| Dose of I3C (mg/kg b.w.) | 4-day treatment[a] Caspase-3 | Caspase-8 | Caspase-9 | 10-day treatment[a] Caspase-3 | Caspase-8 | Caspase-9 |
|---|---|---|---|---|---|---|
| 0[b] | 0.063 ± 0.006 | 0.046 ± 0.011 | 0.037 ± 0.010 | 0.053 ± 0.004 | 0.065 ± 0.035 | 0.053 ± 0.033 |
| 5 | 0.211 ± 0.054[c] | 0.119 ± 0.038[d] | 0.091 ± 0.010[d] | 0.129 ± 0.051[d] | 0.083 ± 0.036 | 0.061 ± 0.024 |
| 25 | 0.105 ± 0.025 | 0.081 ± 0.034 | 0.073 ± 0.051 | 0.192 ± 0.078[c] | 0.118 ± 0.023[d] | 0.106 ± 0.029[d] |
| 250 | 0.123 ± 0.046[d] | 0.103 ± 0.052[d] | 0.081 ± 0.039 | 0.074 ± 0.009 | 0.066 ± 0.032 | 0.050 ± 0.012 |

[a]Treatment of rats and assays of caspase activities are described in Materials and Methods. Data are the mean ± SD from duplicate assays with 4 rats per treatment group.
[b]Vehicle-treated rats.
[c]Statistically significant ($p<0.01$) compared to vehicle-treated group.
[d]Statistically significant ($p<0.05$) compared to vehicle-treated group.

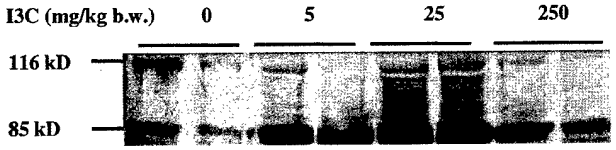


Figure 4. *Western blot analysis of PARP cleavage in the mammary gland of rats treated with I3C for 4 days. Anti-PARP antibody (H-250, Santa Cruz) recognize both the whole PARP (116 kD) and cleaved stable fragment (85 kD). The analysis was carried out with mammary gland of 2 rats in each treatment group.*

Table II. *Effect of treatment of rats with DIM on caspase activities in the mammary gland.*

| Dose of DIM (mg/kg b.w.) | 4-day treatment[a] Caspase-3 | Caspase-8 | Caspase-9 |
|---|---|---|---|
| 0[b] | 0.101 ± 0.038 | 0.036 ± 0.013 | 0.016 ± 0.006 |
| 8.4 | 0.132 ± 0.047 | 0.040 ± 0.008 | 0.017 ± 0.002 |
| 42 | 0.152 ± 0.075 | 0.041 ± 0.005 | 0.018 ± 0.009 |

[a]Treatment of rats and assays of caspase activities are described in Materials and Methods. Data are the mean±SD from duplicate assays with 4 rats per treatment group.
[b]Vehicle-treated rats.

that had developed during or after a 12-week treatment of rats with I3C, β-NF or vehicle were examined. Even though somewhat greater numbers of apoptotic cells were shown by the TUNEL assay in the mammary adenocarcinomas of I3C- or β-NF- than vehicle-treated rats, no statistical differences were found among these groups (Figure 1). The TUNEL assay is considered highly sensitive for detecting DNA strand breaks in cell nuclei (25), but the total cell count in a given field of the tumor section may be subjective and thus lead to variable estimates of the percentage of apoptotic cells.

The apoptotic process in the mammary adenocarcinomas was determined by assays of the activities of caspase-3, caspase-8 and caspase-9 in tumor lysates. Caspase-3 is a downstream effector caspase that cleaves various cytoplasmic and nuclear substrates, resulting in the typical morphological characteristics of apoptosis (23). In addition, caspase-8 and caspase-9 are upstream regulators of caspase-3, and are activated through different pathways. In our study, no significant differences in the activities of the caspases were found in the mammary adenocarcinomas among the treatment groups (Figure 2). At the higher upstream level, the apoptotic process is regulated by interactions of Bax and Bcl-2 genes, in which Bcl-2 protects cells from apoptosis and increased expression of Bax inactivates Bcl-2 during apoptosis (23,26,27). Hence, the assessment of a ratio of Bax to Bcl-2 proteins gives a relevant insight into the apoptotic process. Whereas exposure of MDA-MB-435 breast cancer cells to I3C inhibited cell growth in a dose-dependent manner and induced apoptosis by up-regulation of Bax and down-regulation of Bcl-2 and activation of caspase-3 activity (13), exposure of MCF-7 breast cancer cells to I3C also induced cell growth suppression and apoptosis but not *via* increased expression of Bax or induction of p53 (15). In the mammary adenocarcinomas examined by Western blot analysis in our study, there was no evidence for the increased expression of Bax protein by treatment of rats with I3C or β-NF (Figure 3) and, thus, induction of apoptosis *in vivo*. It is possible that the efficacy *in vivo* of these *in vitro* inducers of apoptosis (7, 12-

15) might have been compromised by their metabolism. Moreover, induction of apoptosis in the mammary adenocarcinomas *in vivo* may be complicated by tumor stage-dependent levels of apoptotic activities. Thus, delineation of the phase of tumorigenesis sensitive to induction of apoptosis and an active form or metabolite of the intended effector of apoptosis require further studies.

Apoptosis in the normal mammary gland was effected by a short-term treatment of rats with I3C in that all three caspase activities were increased up to 3.6-fold after treatment with I3C at 5 and 25 mg/kg b.w. for 4 or 10 days, and caspase-3 and caspase-8 activities were increased ~2-fold after treatment with I3C at 250 mg/kg b.w. for 4 days (Table I). Importantly, the pattern of activation of caspase-3 activity was supported by Western blot analysis of cleavage of PARP (Figure 4), an early marker of apoptosis (13). However, there was no linear association between the extents of I3C-dependent increases in caspase-3, caspase-8 and caspase-9 and the oral dose of I3C administered or duration of treatment. This indicates that kinetics of transformation of I3C *in vivo* are complex and the yields of the individual condensation products formed in the acid pH of stomach vary and may not be proportional to the dose of I3C ingested (24,28).

DIM is reportedly a major product of transformation of I3C *in vivo* (24). Our study examined caspase activities in the mammary gland after a 4-day treatment of rats with DIM at the dose levels equimolar to those of I3C shown to increase these activities. However, treatment with DIM yielded no significant changes in caspase-3, caspase-8 and caspase-9 activities (Table II). It thus appears that the increases in apoptotic activities in the mammary gland observed after treatment with I3C are not due to DIM. Nevertheless, it is possible that suppression of mammary tumor growth after treatment of tumor-bearing rats with DIM and its antiestrogenic activity in MCF-7 cells (19) may involve increased apoptosis in these tumors.

There is evidence that the presence of estrogen protects cells from apoptosis, whereas withdrawal of estrogen induces apoptosis (29,30). Indirectly, our data are consistent with the above reports. A 12-week treatment of rats with I3C at 250 mg/kg b.w. increased ~2-fold the ratio of reductive to oxidative 17β-hydroxysteroid dehydrogenase activities in the mammary adenocarcinomas (31). Hence, an increase in conversion of estrone to a more estrogenic 17ß-estradiol (E2) might lead to stimulation of E2-dependent tumor cell proliferation and protection from apoptosis. Moreover, a 4-day treatment of rats with I3C at 250 mg/kg b.w. increased the capacity of mammary gland microsomes for oxidation of E2, especially to 2-catechol estrogen (31). Thus, a decrease in the levels of E2 might be responsible for increases in apoptotic activity in the mammary gland observed herein. The role of estrogen in apoptosis is also supported by a recent report that a 1-to 3-day treatment of mammary tumor-bearing rats with tamoxifen, an antiestrogen, induced caspase-3 activity and apoptotic cell death in these tumors (32).

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**Suppression of Mammary Gland Carcinogenesis by Post-Initiation Treatment of Rats with Tamoxifen or Indole-3-Carbinol or their Combination**

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A more effective inhibition of growth of human MCF-7 breast cancer cells was achieved with a combination of tamoxifen (TAM) and indole-3-carbinol (I3C) than with either agent alone (Cover et al *Cancer Res* 59: 1244, 1999). Our study examined the post-initiation effects of TAM, I3C or TAM+I3C on mammary gland tumorigenesis induced in seven week-old female Sprague-Dawley rats with one oral dose of 7,12-dimethylbenz[*a*]anthracene (DMBA) at 65 mg/kg of body weight (bw). Starting two weeks after DMBA, rats were treated with TAM (10 µg/rat) or 10% ethanol in olive oil (Veh I at 50 µL/rat) by s.c. injections, and/or with I3C (250 mg/kg bw) or 20% ethanol in olive oil (Veh II at 2.5 mL/kg bw) by oral gavages, three times per week, for up to 20 weeks. Four groups included: 1) Veh I+Veh II ($n$=21)-; 2) TAM+Veh II ($n$=18)-; 3) Veh I+I3C ($n$=19)-; and 4) TAM+I3C ($n$=23)-treated rats. Since all tumors in group 1 and the majority of those in groups 2, 3 and 4 were malignant (mammary adenocarcinomas and ductal carcinomas *in situ*), statistical analyses of tumor incidence and latency, multiplicity and mass concerned malignant tumors only. The median latent period of tumors was 72, 119, 70 and 112 days in groups 1, 2, 3 and 4, respectively. Since the differences between groups 2 and 1 ($P$=0.0023), 4 and 1 ($P$=0.0008), and 4 and 3 ($P$=0.0356) were significant, the observed suppression of mammary carcinogenesis through the increased tumor latency was due to TAM. At 112 days after DMBA, the estimated malignant tumor incidences were 84, 46, 65 and 51% in groups 1, 2, 3 and 4, respectively. The mean number of malignant tumors per rat ± SD in group 2 (0.94±0.94), 3 (1.47±1.02) or 4 (0.61±0.72) was significantly different ($P$=0.0007, $P$=0.0493 or $P$< 0.0001, respectively) from that in group 1 (2.24 ± 1.48), and that between group 4 and 3 was also different ($P$= 0.0014). Likewise, the mean number of malignant tumors per tumor (malignant+benign)-bearing rat ± SD in group 2 (1.42±0.79), 3 (1.75±0.86) or 4 (1.08±0.64) was significantly different ($P$=0.0009, $P$=0.0145 or $P$<0.0001, respectively) from that in group 1 (2.61 ± 1.24), and that between group 4 and 3 was also different ($P$=0.0122). Thus, the decrease in malignant tumor multiplicity achieved by treatment of rats with TAM was greater than that with I3C, and TAM in combination with I3C amplified the decrease effected by the latter. The mean tumor weight (g) per rat ± SD for group 2 (0.49±0.65) or 4 (0.59±1.47) was statistically different ($P$=0.0251 or $P$=0.0034, respectively) from that of group 1 (1.55±1.93), and that of group 4 was also different ($P$=0.0338) from group 3 (0.79±1.07). Hence, the decrease in malignant tumor weight was achieved only by treatment of rats with TAM. In conclusion, post-initiation treatment of rats with TAM at very low doses effectively suppresses mammary gland carcinogenesis as shown by the increase in malignant tumor latency and decreases in tumor multiplicity and mass. The suppressing effect of I3C alone is limited to the decrease in the malignant tumor multiplicity, and this is enhanced by co-treatment with TAM. Importantly, administration of I3C at the dose level that maintains upregulation of oxidative metabolism of 17ß-estradiol and estrone in the liver, does not diminish the suppressing effects of TAM on mammary carcinogenesis.