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TITLE: Expression of Metabolic and Apoptotic Genes During  
Treatment With Chemopreventive Agents for Breast Cancer

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## 1. OBJECTIVE

Indole-3-carbinol (I3C), a compound found in cruciferous vegetables, has been shown to suppress estrogen-dependent cancers. Experiments are aimed at identifying the metabolic and apoptotic genes underlying the mechanisms of action of I3C in experimentally induced mammary carcinogenesis. The specific aims include determination of: 1) the effects of short-term (up to 2 weeks) treatment of rats with I3C at three dose levels (5, 25, and 250 mg/kg body weight (bwt)) on mRNA expression of cytochrome P450s (CYPs) in the liver and mammary gland and apoptotic activities in the mammary gland; and 2) the effects of long-term (up to 16 weeks) post-carcinogen treatment of rats with I3C and/or tamoxifen (TAM) on mammary tumorigenesis and apoptosis in the mammary gland and mammary tumors.

## 2. STUDIES AND RESULTS

**Under aim 1**, treatments of female Sprague-Dawley rats included three dose levels of I3C, i.e. 5, 25, and 250 mg/kg of bwt, 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  $-80^{\circ}\text{C}$ . CYP mRNA expression in the liver and mammary gland, CYP probe activities, and CYP-dependent metabolism of  $17\beta$ -estradiol (E2) and estrone (E1) by liver microsomes were examined. The methods included: semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) to estimate CYP mRNA transcripts, spectrophotometric assay of P450:carbon monoxide complex, spectrofluorimetric assays of CYP specific alkoxyresorufin *O*-dealkylase activities, and HPLC/radiometric assays of metabolites from  $^{14}\text{C}$ -labeled E2 and E1. The mRNA transcripts for hepatic CYP1A1, 1B1, and 2B1/2 and mammary CYP1A1 were upregulated after treatment with I3C at 250 mg/kg. This treatment also increased the capacity of liver microsomes to metabolize E2 to 2-OH-E2, 2-OH-E1, 6- $\alpha$ -OH-E2, 6- $\beta$ -OH-E2, estriol and 15- $\alpha$ -OH-E2, and E1 to 2-OH-E1, 2-OH-E1, 2-OH-E2, 6( $\alpha$ + $\beta$ )-OH-E1 and 6 $\alpha$ -OH-E2. The increases of CYP-dependent activities and rates of estrogen metabolite formation were smaller after 10 than 4 treatments. The results indicate that alterations in CYP complement and metabolite composition from E2 and E1 are I3C dose- and treatment duration-dependent and suggest that biological activity of I3C administered at low dose levels does not involve the changes in estrogen metabolism.

Further, apoptosis in the mammary gland after the 4- and 10-day treatments of rats with I3C at the above dose levels was examined. Total protein was isolated from mammary gland, quantitated, and used to measure the activities of caspases 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. The relative absorbance at 405 nm was determined on plate reader. Statistical analysis was performed with ANOVA. Four-dose treatment with I3C at 5 mg/kg bwt induced significantly ( $P < 0.05$ ) the activities of caspase-3 (3.3-fold), caspase-8 (2.5-fold), and caspase-9 (2.5-fold). After 10-dose treatment with I3C at 25 mg/kg bwt, the activities of caspase-3 (3.6-fold), caspase-8 (1.8-fold), and caspase-9 (2-fold) were induced significantly ( $P < 0.05$ ). To elucidate and confirm dose-related effects of I3C on caspase activities, other apoptotic genes (e.g. PARP, DFF45) will be assayed to determine the mechanisms by which I3C may affect mammary gland before and during carcinogenesis process.

**Under aim 2**, The methods for assays of apoptotic activities in mammary tumors were evaluated using the tissues from the Mentor's previous study (*Cancer Letters* 160: 209-218, 2000). Thus, apoptotic activities in 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced mammary adenocarcinomas in female Sprague-Dawley rats, and potential modulation of apoptosis by treatment of rats with indole-3-carbinol (I3C) at 250 mg/kg or  $\beta$ -naphthoflavone ( $\beta$ -NF) at 20 mg/kg of body weight in ethanol:corn oil (2:3), by oral gavage, three times per week for up to 12 weeks starting 3 weeks after DMBA (one oral dose of 20 mg per 7-week-old rat) were examined. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was performed in tumor sections prepared from paraffin-embedded tissues. The number of apoptotic cells and total number of cells in each field (6 to 8 fields per section) were counted using light microscopy and Image-Plus program. The percentages of apoptotic cells were  $2.95 \pm 1.23\%$  ( $n = 10$ ),  $4.93 \pm 3.31\%$  ( $n = 12$ ), and  $3.61 \pm 1.72\%$  ( $n = 11$ ) in tumors from the vehicle-, I3C- and  $\beta$ -NF-treated rats, respectively. Caspase-3, caspase-8, and caspase-9 activities were determined with *N*-acetyl-Asp-Glu-Val-Asp- $p$ -nitroanilide, *N*-acetyl-Ile-Glu-Thr-Asp- $p$ -nitroanilide and *N*-acetyl-Leu-Glu-His-Asp- $p$ -nitroanilide as the specific substrate, respectively, using total tumor protein and colorimetric (absorbance at 405 nm) development. The relative absorbance for caspase-3 activity was  $0.306 \pm 0.154$  ( $n = 16$ ) for vehicle-,  $0.321 \pm 0.158$  ( $n = 20$ ) for I3C- or  $0.372 \pm 0.136$  ( $n = 15$ ) for  $\beta$ -NF-treated rats. However, the higher activities of caspase-3 in tumors of  $\beta$ -NF- or I3C-treated rats were not significantly different from that of the vehicle-treated group ( $P = 0.11$ ). Likewise, the relative absorbances for caspase-8 activity,  $0.225 \pm 0.083$  ( $n = 12$ ) for vehicle-,  $0.178 \pm 0.090$  ( $n = 12$ ) for I3C- or  $0.255 \pm 0.075$  for  $\beta$ -NF-treated rats, and caspase-9 activity,  $0.152 \pm 0.100$  ( $n = 10$ ) for vehicle-,  $0.175 \pm 0.075$  ( $n = 8$ ) for I3C- or  $0.243 \pm 0.105$  ( $n = 8$ ) for  $\beta$ -NF-treated rats were not significantly different among the groups. Protein expression levels of the antiapoptotic gene, Bcl-2, and apoptotic gene Bax, were determined by Western blot analysis using total tumor protein. The pattern of expression of Bcl-2 or Bax was variable, and it did not follow the reported upregulation of Bax by I3C in breast cancer cells *in vitro*. Although I3C or  $\beta$ -NF administered before DMBA suppresses mammary tumorigenesis, these compounds in the post-carcinogen regimen do not significantly change the outcome of tumorigenesis and apoptotic activities in mammary tumors.

### 3. PUBLICATION

#### Abstracts:

- 1) Zhang, X. and Malejka-Giganti, D. Detection of apoptosis in rat mammary adenocarcinomas. In: Era of Hope Department of Defense Breast Cancer Research Program Meeting, Orlando, FL, Sep 25-28, 2002.
- 2) Beland, F.A., Churchwell M.I., Doerge, D.R., Malejka-Giganti, D., Zhang, X., Divi R.L., Poirier, M.C., Costa, G.G., Marques, M.M. High performance liquid chromatography electrospray ionization tandem mass spectrometry analysis of tamoxifen DNA adducts in rats, monkeys, and humans. *Proc Amer Assoc Cancer Res.* 43: 343,2002.

### 4. APPENDICES

Abstract for DoD Meeting: "DETECTION OF APOPTOSIS IN RAT MAMMARY ADENOCARCINOMAS "

## DETECTION OF APOPTOSIS IN RAT MAMMARY ADENOCARCINOMAS

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Induction of apoptosis is one approach to suppression of cancer. Extensive efforts have focused on induction of apoptosis in cancer cells *in vitro* and elucidation of the mechanisms of apoptosis by antitumor agents. However, data on drug-modulated apoptotic activities in animal tumor models are scarce. Our study examined apoptotic activities in 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced mammary adenocarcinomas in female Sprague-Dawley rats, and potential modulation of apoptosis by treatment of rats with indole-3-carbinol (I3C) at 250 mg/kg or  $\beta$ -naphthoflavone ( $\beta$ -NF) at 20 mg/kg of body weight in ethanol:corn oil (2:3), by oral gavage, three times per week for up to 12 weeks starting 3 weeks after DMBA (one oral dose of 20 mg per 7-week-old rat). Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was performed in tumor sections prepared from paraffin-embedded tissues. The number of apoptotic cells and total number of cells in each field (6 to 8 fields per section) were counted using light microscopy and Image-Plus program. The percentages of apoptotic cells were  $2.95 \pm 1.23\%$  ( $n = 10$ ),  $4.93 \pm 3.31\%$  ( $n = 12$ ), and  $3.61 \pm 1.72\%$  ( $n = 11$ ) in tumors from the vehicle-, I3C- and  $\beta$ -NF-treated rats, respectively. Caspase-3 and caspase-9 activities were determined with *N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide and *N*-acetyl-Leu-Glu-His-Asp-*p*-nitroanilide as the specific substrate, respectively, using total tumor protein and colorimetric (absorbance at 405 nm) enzyme-linked immunosorbent assays (ELISA). The relative absorbance for caspase-3 activity was  $0.306 \pm 0.154$  ( $n = 16$ ) for vehicle-,  $0.321 \pm 0.158$  ( $n = 20$ ) for I3C- or  $0.372 \pm 0.136$  ( $n = 15$ ) for  $\beta$ -NF-treated rats. However, the higher activities of caspase-3 in tumors of  $\beta$ -NF- or I3C-treated rats were not significantly different from that of the control group ( $p = 0.11$ ). Likewise, the relative absorbance for caspase-9 activity of  $0.152 \pm 0.100$  ( $n = 10$ ) for vehicle-,  $0.175 \pm 0.075$  ( $n = 8$ ) for I3C- or  $0.243 \pm 0.105$  ( $n = 8$ ) for  $\beta$ -NF-treated rats was not significantly different among the groups. Protein expression levels of the antiapoptotic gene, Bcl-2, and apoptotic gene Bax, were determined by Western blot analysis using total tumor protein. The pattern of expression of Bcl-2 or Bax was variable, and it did not follow the reported upregulation of Bax by I3C in breast cancer cells *in vitro*. In contrast to inhibition of mammary tumorigenesis by I3C or  $\beta$ -NF administered before DMBA, these compounds used in the post-carcinogen regimen do not significantly change the outcome of tumorigenesis observed in our previous study and apoptotic activities in mammary tumors shown herein.

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