

Effects of 5,6-benzoflavone, indole-3-carbinol (I3C) and diindolylmethane (DIM) on chemically-induced mammary carcinogenesis: Is DIM a substitute for I3C?

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Abstract. The abilities of 5,6-benzoflavone (5,6-BF, a synthetic flavonoid), indole-3-carbinol (I3C, a plant derived product) or diindolylmethane (DIM, a condensation product of I3C) to alter the induction of mammary cancers induced by the carcinogens 7,12-dimethylbenzanthracene (DMBA) or N-methyl-N-nitrosourea (MNU) were evaluated. Interestingly, the first two agents act as aryl hydrocarbon receptor (AhR) agonists, while DIM does not. The agents were initially examined for their ability to inhibit DMBA-induced mammary carcinogenesis. Agents were administered for 14 days starting 7 days prior to a single dose of the carcinogen. Evaluated over an extensive range of doses (165, 550 and 1650 ppm in the diet), 5,6-BF caused a dose-dependent decrease of mammary cancers. In addition, 5,6-BF at doses of 1650 and 165 ppm in the diet blocked the induction of DMBA-induced DNA adducts in the mammary gland by approximately 85% and 45%, respectively. In contrast, DIM (180 or 20 mg/kg BW/day) failed to block induction of DMBA tumors. The effect of these agents on the promotion/progression phase of carcinogenesis using the MNU mammary cancer model was also determined. 5,6-BF (1650 or 165 ppm in the diet), I3C (180 or 60 mg/kg BW/day administered by gavage), or DIM (180 or 60 mg/kg BW/day by gavage) were initiated 5 days after the administration of MNU, and continually thereafter. 5,6-BF decreased MNU-induced mammary tumor multiplicity by 40-60%. I3C reduced tumor multiplicity at the high dose, while DIM at either dose had minimal effects on tumor multiplicity. Thus, 5,6-BF and I3C were highly effective against initiation of DMBA-induced

mammary carcinogenesis, and were also effective against MNU-induced tumors during the promotion/progression phase of carcinogenesis. In contrast, DIM had minimal effects in either model; arguing that administration of DIM is not analogous to administration of I3C.

Introduction

5,6-benzoflavone (5,6-BF) is a synthetic flavonoid that has been used for many years as a relatively non-toxic Ah receptor agonist. The compound binds to the Ah receptor (AhR) and induces transcriptional activation of a wide variety of phase I/II drug metabolizing enzymes (1,2). 5,6-BF was examined as a potential chemopreventive agent as early as the mid 1970's, and proved to be capable of inhibiting 7,12-dimethylbenzanthracene (DMBA)-induced mammary, lung, and aflatoxin B1-induced hepatocarcinogenesis (3-5).

The plant product indole-3-carbinol (I3C) similarly activates the AhR, and is found at high levels in cruciferous plants. I3C has also been examined as a potential chemopreventive agent and has been shown to be effective in a wide variety of carcinogen-induced tumor models (6-9). It has been proposed that the efficacy of many of the so-called blocking agents, such as 5,6-BF or I3C, on cancers initiated by procarcinogens (DMBA, aflatoxin, etc.) may be due to inhibition of the activation of carcinogens to their proximate moiety (7,8). In agreement with this hypothesis, it has been shown that a number of agents that inhibit DMBA binding to DNA simultaneously inhibit DMBA-induced tumorigenesis (10-12). In addition to its effects in altering carcinogen metabolism, I3C has been shown to inhibit carcinogenesis in certain hormonally-related tumor models; e.g., mammary gland and endometrium of the uterus that do not involve carcinogen metabolism (13,14). In fact, certain other AhR agonists have similarly been shown to affect direct-acting mammary carcinogens. Although the anti-tumor effects of these AhR agonists have been primarily attributed to altered metabolism of endogenous estrogens (i.e., estradiol), it has also been suggested that I3C or certain products resulting from breakdown or polymerization of I3C may have more direct anti-estrogenic effects (15-17).

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I3C is rapidly modified in the stomach to produce a wide variety of products including diindolylmethane (DIM). Although various condensation products, including indolo-(3,2b)-carbazole, interact with the Ah receptor (18), DIM itself does not bind to this Ah receptor; but, nevertheless, has been shown to have antagonistic effects both with regards to the androgen and estrogen receptors (17,19).

The specific objectives of the present studies were to determine: a) the dose response for 5,6-BF mediated inhibition of DMBA-induced mammary carcinogenesis, and its relationship to preventing formation of DMBA-related adducts in rat mammary epithelial cells; b) whether 5,6-BF, I3C and DIM can affect the tumor promotion/progression phase in the MNU-induced mammary cancer model; and c) if DIM, which is chemically stable, is a substitute for the potential chemopreventive effects of the chemically unstable I3C.

Materials and methods

Chemicals. DMBA, 5,6-BF and I3C were obtained from Sigma Chemical Co. (St. Louis, MO). DIM was obtained from The National Cancer Institute, Division of Cancer Prevention Repository. MNU was obtained from Ash Stevens (Detroit, MI).

Experimental animals. Female Sprague-Dawley rats were obtained from Harlan Sprague-Dawley, Inc. (virus-free colony number 205) at 4 weeks of age, and were placed on Teklad (4%) diet. The rats were housed 5/cage in a room maintained at 22±2°C and artificially lighted 12 h/day. Animals were allowed free access to the diet and drinking water throughout the duration of the experiments. All animal experiments were conducted in facilities at the University of Alabama at Birmingham following the procedures approved by the Institutional Animal Care and Use Committee.

Preparations of various agents. I3C, which was found to be unstable in the diet, was administered by daily (7x/week) gavage. The compound was dissolved in degassed ethanol, and then mixed with corn oil to the appropriate dose level just prior to dosing the rats (gavage volume = 0.5 ml) (20). The ratio of ethanol to corn oil was 40:60, v/v. DIM was prepared and administered using procedures identical to I3C, except the vehicle was ethanol:corn oil (10:90, v/v). 5,6-BF was mixed directly into the diet using a Patterson-Kelly blender with intensifier bar.

Treatment with various agents in the DMBA-induced model of mammary carcinogenesis. Animals were treated as previously described (20). In brief, rats were treated with the indicated levels of 5,6-BF (1650, 550 and 165 ppm in diet), DIM (180 or 20 mg/kg BW/day by gavage), or I3C (180 mg/kg BW/day) starting at 43 days of age. They were kept on these compounds until 57 days of age. Control animals were administered Teklad 4% diet plus the appropriate vehicle. One week following the initiation of the treatments rats were administered DMBA (12 mg) by gavage (50 days of age). Rats were weighed weekly, palpated for mammary tumors twice per week, and checked daily for signs of toxicity. The studies were terminated approximately 4 months following DMBA

administration. When the studies were terminated, mammary tumors were removed, and examined histopathologically.

Animals and treatments for DMBA adduct studies. Female Sprague-Dawley rats were obtained from Harlan Sprague-Dawley, Inc. and were housed as outlined above. Three groups of rats (5/group) were established as follows: group 1, DMBA; group 2, DMBA plus 5,6-BF (1650 mg/kg diet); group 3, DMBA plus 5,6-BF (165 mg/kg diet). DMBA-treated rats received a single administration of the carcinogen by gavage (12 mg/kg BW) at 67 days of age. The 5,6-BF treated groups received the agent for 7 days before DMBA treatment. Rats were sacrificed by CO₂ asphyxiation 48 h after DMBA administration. Mammary glands were rapidly removed and stored in liquid nitrogen (21).

DNA adduct analyses in rat mammary epithelial cells. The samples were shipped from the University of Alabama at Birmingham on dry ice to the University of Genoa, where they were immediately stored at -80°C. Fat-free mammary epithelial cells were prepared (22) and DNA adducts were examined as previously described (12).

Determination of levels of RNAs coding for liver phase I drug metabolizing enzymes. At 60 days of age, female Sprague-Dawley rats were treated with 5,6-BF (1650 ppm), DIM (180 mg/kg BW) or vehicle for a period of 7 days. After 7 days, rats were sacrificed and the livers processed for use in microarrays as previously described (23,24). The expressions of CYP 1A1 and CYP 1B1 were determined in the livers of all rats.

Treatment with various agents in the MNU-induced model of mammary carcinogenesis. Rats were housed as described above for the DMBA model. At 50 days of age, they were injected IV (via the jugular vein) with MNU (50 mg/kg BW) (20). Rats (15/group) were administered the doses of 5,6-BF (1650 or 165 ppm in the diet), I3C (180 or 60 mg/kg BW/day by gavage), or DIM (180 or 60 mg/kg BW/day by gavage) beginning 5 days following the administration of MNU and continuing until the end of the study. Rats were weighed weekly, and palpated for mammary tumors twice per week. The studies were terminated approximately 4 months following MNU administration. All tumors were histologically classified.

Statistical analysis. Chemopreventive effects of I3C or 5,6-BF on mammary cancer incidence and latency were determined employing the log-rank analysis, while differences in cancer multiplicity were determined employing the Armitage test (25).

Results

Effects of agents on DMBA-induced mammary carcinogenesis. Rats were administered 5,6-BF in the diet or DIM by gavage beginning at 43 days of age and ending at 57 days of age (Fig. 2). At 50 days of age, rats were administered a single dose of the carcinogen DMBA. 5,6-BF caused a dose-dependent decrease in DMBA-induced mammary carcinogenesis (Fig. 2A). The highest dose of 5,6-BF (1650 ppm) profoundly inhibited DMBA-induced mammary carcinogenesis; however,

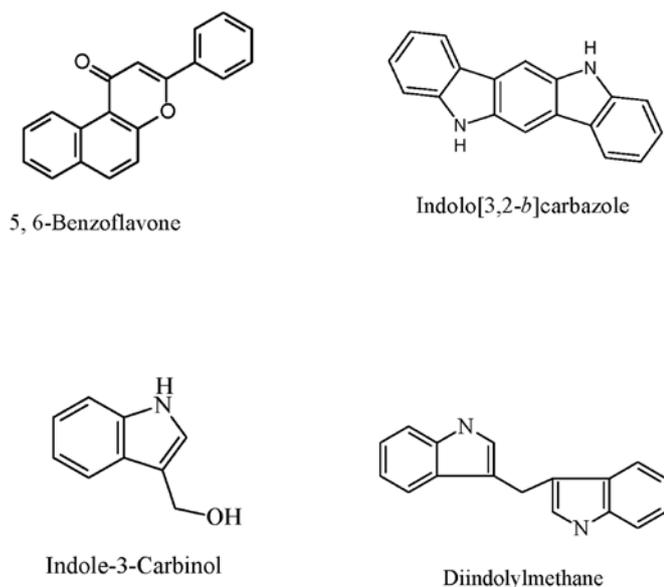


Figure 1. Structures of agents.

Table I. Levels of DMBA ³²P-post-labeled DNA adducts in mammary epithelial cells and effects of treatment with 5,6-benzoflavone.

Autoradiographic spot	Adducts/10 ⁸ /nucleotides		
	DMBA	DMBA + 5,6-BF (1650 ppm)	DMBA + 5,6-BF (165 ppm)
1	18.6±6.5	2.7±1.2 ^a	10.3±3.7 ^b
2	3.8±1.4	0.8±0.8 ^b	2.5±0.9
3	2.4±1.3	0.4±0.4 ^a	2.0±0.7
4	1.7±1.1	0.3±0.2 ^b	1.2±0.9
5	3.2±1.9	0.6±0.6 ^a	1.8±1.0
Total	29.7±12.1	4.9±3.0 ^a	16.6±9.2 ^b

DMBA (12 mg/kg BW) administered once by gavage to female Sprague-Dawley rats. 5,6-BF administered in the diet. Values are mean ± SD; n=5. Duplicate analyses performed on each sample. ^aP<0.01, ^bP<0.05 compared to DMBA alone; Student's t-test for unpaired data.

even the low dose of 165 ppm inhibited formation of DMBA-induced mammary cancers by almost 50%. In contrast, DIM failed to alter the induction of mammary tumorigenesis when given for this time period (Fig. 2B).

Effects of 5,6-BF treatment on levels of DMBA-DNA adducts in rat mammary epithelial cells. Levels of DMBA-DNA adducts were examined as previously described (12) in mammary epithelial cells by employing a ³²P-post-labeling procedure; which has a sensitivity level of 0.1 adducts/10⁸ nucleotides (Table I). Administration of a single dose of DMBA (12 mg/kg BW) by gavage resulted in the formation of multiple DNA adducts in mammary epithelial cells. The autoradiograph spots observed during ³²P-post-labeling analyses likely were from reactions between anti-DMBA-dihydrodiol

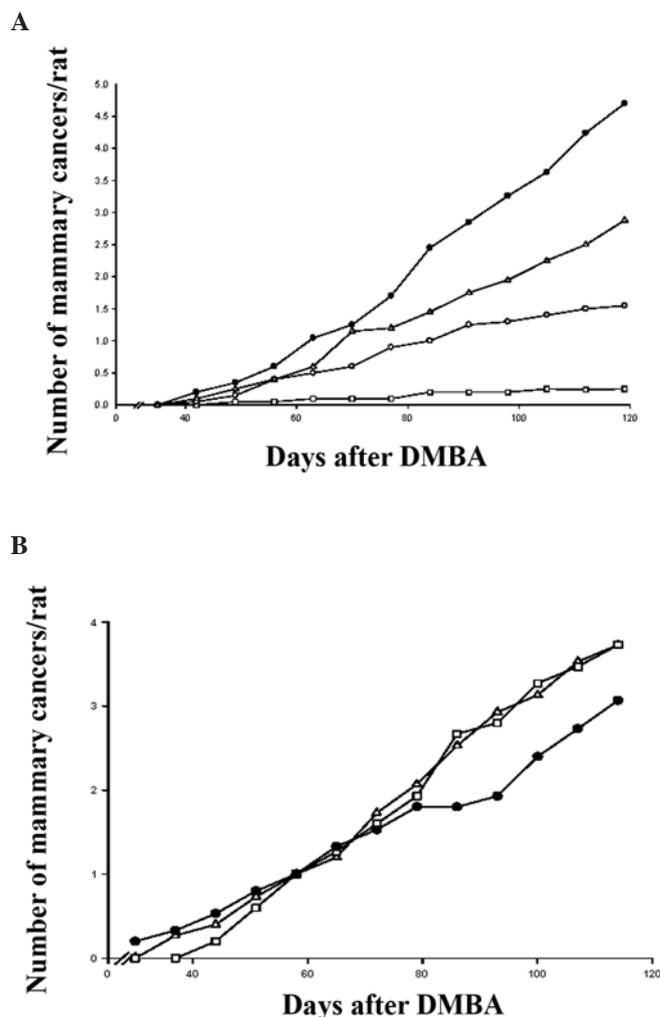


Figure 2. Effects of agents on the time of appearance of DMBA-induced mammary cancers in female Sprague-Dawley rats. (A), 5,6-benzoflavone: □, 1650 ppm; ○, 550 ppm; △, 165 ppm; ●, none. (B), Diindolylmethane: □, 180 mg/kg BW/day; ○, 20 mg/kg BW/day; ●, none.

epoxide and either dG (spots 1 and 5) or dA (spot 2), and between syn-DMBA-dihydrodiol epoxide and either dG (spot 3) or dA (spot 4) (25). Administration of 5,6-BF in the diet immediately prior to and following DMBA administration resulted in a significant and marked inhibition of all formed DNA adducts by approximately 85%. The 5 autoradiographic spots were reduced by treatment with the chemopreventive agent to a similar extent; i.e., by 85.5, 78.9, 83.3, 82.4 and 81.3%, respectively. Treatment of rats with 165 ppm 5,6-BF decreased overall DNA adduct formation roughly 45% and decreased the levels of individual adducts 40-50%.

Effects of 5,6-BF or DIM on the induction of CYP 1A1 and CYP 1B1. The ability of 5,6-BF and DIM to induce CYP 1A1 and CYP 1B1 was examined (Fig. 3). As can be readily observed, the 5,6-BF (1650 ppm in the diet) profoundly induced the RNAs coding for both of these enzymes in the livers of treated rats. In contrast, DIM failed to induce levels of RNAs coding for the genes of either of these CYPs.

Effects of agents on MNU-induced mammary carcinogenesis. Rats were treated with 5,6-BF, I3C or DIM starting at 55 days

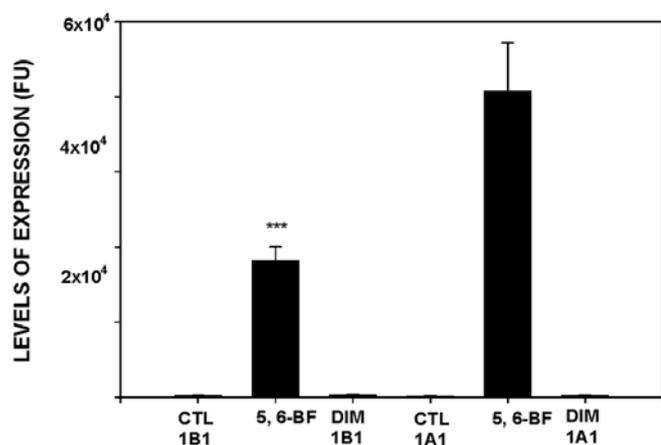


Figure 3. Induction of CYP 1A1 and CYP 1B1 in livers of rats treated short-term (7 days) with 5,6-benzoflavone (1650 ppm) or diindolymethane (180 mg/kg BW). CTL, controls. RNA was isolated and processed as per Materials and methods.

of age; 5 days following treatment with MNU (Fig. 4). The higher dose (1650 ppm) of 5,6-BF decreased tumor multiplicity by 40%. Interestingly, the lower dose of 5,6-BF (165 ppm) decreased tumor multiplicity by almost 50%. At the higher dose of 5,6-BF, liver to body weight ratios increased approximately 20% (data not shown). I3C at 180 mg/kg BW/day reduced tumor multiplicity by 65%. The higher dose of I3C significantly increased liver to body weight ratios by 28%. Finally, no significant effect on tumor multiplicity was observed in rats treated with either dose of DIM. These agents were examined for their effects on the estrus cycles of the rats. None of the agents altered estrus cycles.

Discussion

I3C is found at high levels in a variety of plants, while 5,6-BF is a synthetic product. Both of these agents or their metabolites are known to interact with the Ah receptor (1,2,20,23). These compounds induce both phase I (oxidative, generally P450 mediated) and phase II (conjugation) drug metabolizing enzymes. When examining the CYP genes, 5,6-BF appears to be a typical 'dioxin type' inducer; increasing levels of the various CYP1 super family (e.g., CYP 1A1, CYP 1A2, and CYP 1B1), and actually decreasing activities of certain other CYP proteins (e.g., CYP 3A). In contrast, I3C is quite unusual since it induces the CYP1 superfamily as well as phenobarbital inducible CYPs 2B and 3A (20,27). Presumably, this dichotomy is due to the fact that certain of the condensation products [e.g., indolo(3,2-b)carbazole], rather than I3C itself, interact with the Ah receptor (19) while I3C mediates the phenobarbital-like response. In addition to inducing these CYP proteins, both of these compounds induce a wide range of phase II drug metabolizing enzymes (glutathione transferases, glucuronyl transferases, sulfotransferases, etc.) (20,27). Therefore, any attempt to determine the relative role of induction of CYPs, various conjugating enzymes, etc. in their ability to inhibit the efficacy of various pro-carcinogens is quite complex. Further complicating such an approach is the fact that these compounds, or their metabolites, are direct inhibitors of various purified

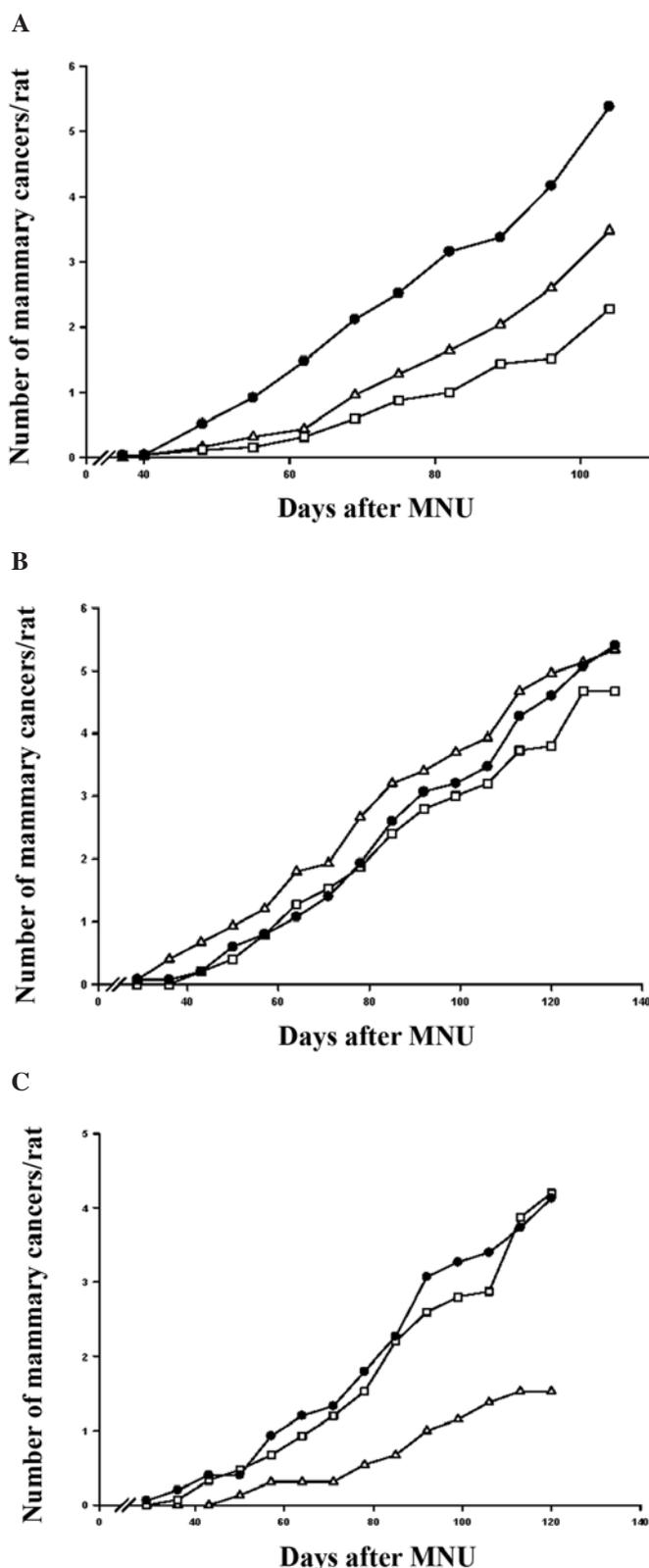


Figure 4. Effects of agents on the time of appearance of MNU-induced mammary cancers in female Sprague-Dawley rats. (A), 5,6-benzoflavone: Δ, 1650 ppm; □, 165 ppm; ●, none. (B), Diindolymethane: □, 180 mg/kg BW/day; Δ, 60 mg/kg BW/day; ●, none. (C), Indole-3-carbinol: Δ, 180 mg/kg BW/day; □, 60 mg/kg BW/day; ●, none.

cytochromes P450s; including those cytochromes which they induce. In contrast, DIM failed to induce RNA coding for

either CYP 1A1 or CYP 1B1. This is compatible with prior studies showing that DIM, unlike certain other I3C condensation products, was not an agonist for the Ah receptor (17,27). Although at very high doses in cell culture, investigators have reported limited Ah receptor agonist activity (28).

5,6-BF has previously been evaluated in the DMBA-induced rat mammary tumor model. The studies of Wattenberg and Leong (3), reported almost 30 years ago, employed a single large dose of 5,6-BF while a recent study employed repeated gavage dosing (29). In a previous set of experiments (data not shown), we had examined relatively high, albeit non-toxic, doses of 5,6-BF (3300 and 1650 ppm in the diet), and found both doses to be profoundly effective in reducing tumor formation (>90%). The present experiments were designed to determine the dose response to 5,6-BF and possibly a 'no effect' dose. Despite the fact that the dose of 5,6-BF was decreased x20 (3300 vs. 165 ppm), we failed to achieve an ineffective dose. In fact, almost a 50% decrease in tumor multiplicity at the lowest dose of 5,6-BF was still observed.

Interestingly, in the DMBA-induced rat mammary model mixed inducers such as 5,6-BF or I3C appear to optimally yield more striking effects than can be achieved with optimal doses of most of the relatively specific phase II inducers such as 1,2-dithiolthione or oltipraz (23). DIM, which does not interact with the Ah receptor and does not induce the various phase I and II drug metabolizing enzymes, failed to inhibit DMBA-induced mammary carcinogenesis. The general hypothesis has been that these alterations in DMBA-induced carcinogenesis are likely to reflect differences in DMBA-induced DNA damage in the target tissue (7,8,12). Indeed, it was found that 5,6-BF (1650 and 165 ppm) decreased DMBA-induced DNA adducts in mammary epithelial tissue by 85 and 45%, respectively. Thus, the efficacy of 5,6-BF and I3C in inhibiting DMBA-induced carcinogenesis is presumably associated with their interaction with the Ah receptor and their ability to induce various CYP genes as well as glutathione and UDP. UDP glucuronyl transferases are associated with their activity in this model.

As stated above, the fact that certain of the mixed inducers can alter carcinogen metabolism is no surprise. However, there has been evidence for a long time that certain of these agents can inhibit induction of hormonally-related cancers (breast, endometrial) independently of carcinogen metabolism (13,14,20). We previously showed that I3C decreased tumor multiplicity by 50-60% (20). One criticism of that work was that I3C was administered prior to the carcinogen. Therefore, in order to examine the effect of this chemopreventive agent on the promotion/progression phase of mammary carcinogenesis, we initiated chemopreventive treatment in the present study five days following MNU administration. Under these circumstances, the highest dose of I3C (180 mg/kg BW/day) caused a 65% decrease in tumor multiplicity, while the lowest dose of I3C (60 mg/kg BW/day) had no effect. Interestingly, and somewhat unexpectedly, we did not find that treatment with DIM decreased MNU-induced mammary cancer formation. Prior results by Safe and coworkers (17) had found a substantial inhibitory effect of DIM when administered late in the DMBA model of tumorigenesis. Since tumors from both the DMBA model and the MNU model are primarily ER-positive and responsive to multiple hormonal agents, we had anticipated a decrease in

mammary cancers. One difference is that Safe *et al* (17) used pure DIM, while we used a preparation that had certain inert components as well. Thus, we do not have a clear basis for the apparent discrepancy, but found this lack of efficacy in at least two studies. Finally, we examined the efficacy of I3C and DIM given in conjunction with a suboptimal dose of the SERM, tamoxifen (data not shown). Tamoxifen is approved for prevention of invasive breast cancer in postmenopausal women. We failed to observe any synergy between either of these agents and suboptimal dose of tamoxifen in agreement with the results of Malejka-Giganti *et al* (30).

To confirm the general applicability of the finding with indole-3-carbinol, the effects of 5,6-BF administered during the promotion/progression stage of MNU-induced mammary carcinogenesis was examined. Two doses of 5,6-BF were employed (1650 and 165 ppm in the diet), and it was found that they reduced tumor multiplicity 40-60%. There are a variety of possible mechanisms whereby I3C, 5,6-BF, or related compounds which interact with the Ah receptor might accomplish their effects on mammary tumorigenesis. Bradlow *et al* (13), examining I3C, initially hypothesized that production of 2-OH-estradiol and decreased production of 16-OH-estradiol were associated with the preventive efficacy of these agents. However, besides being a relatively weak estrogen, 2-OH-estradiol serves as a precursor to 2-methoxyestradiol via O-methyltransferases, and this latter compound has both anti-angiogenic and anti-proliferative activities (16). In addition, Safe and coworkers (17) have shown that compounds which interact with the Ah receptor have some direct antagonistic effects with regard to the estrogen receptor; and may thereby block expression of various target genes which are themselves modulated by estrogen levels. In contrast to the efficacy of the Ah receptor agonists in both models, we failed to observe inhibition of tumors in either model treated with DIM. This demonstrates that although DIM may be the major condensation product of I3C, it does not appear to be the primary determinant of the efficacy of I3C.

Despite certain promising aspects of this class of compounds, a variety of questions still arise. First, there is the general fear that induction of phase I drug metabolizing enzymes may increase activation of certain carcinogens. Although clear evidence for this has been demonstrated for preferential activation of various carcinogens *in vitro*, the data with regards to increased activation *in vivo* have not been as convincing (30). In contrast, these agents have been effective in inhibiting carcinogenesis or DNA adduct formation by various carcinogens, including DMBA, aflatoxin B1, MEIQ, NNK and cigarette smoke. Second, I3C has proven to be a liver carcinogen, at least in rainbow trout (31). However, the extrapolation of hepatic carcinogenicity between species has been notoriously difficult. Despite these questions, this class of compounds appears to have significant efficacy in a variety of tumor models. The profound efficacy in many models that involve metabolism of pro-carcinogens warrants further investigation in chemoprevention models. Finally, the Ah receptor agonists have recently been shown to have efficacy in preventing colon cancer development. Their efficacy is based on the fact that the agonist-Ah receptor complex enhance the degradation of β -catenin. β -catenin accumulation is the major pathway by which truncating APC mutations enhance colon cancer (32).

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