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13. ABSTRACT *(Maximum 200 Words)*

Diindolylmethane (DIM) is formed by acid catalyzed dimerization of indole-3-carbinol, and both compounds inhibit formation and/or growth of mammary tumors in rodents. In this study, we have investigated the aryl hydrocarbon receptor (AhR) agonist activity and inhibitory AhR-estrogen receptor crosstalk induced by the following methyl-substituted DIMs: 1,1'-dimethyl-; 2,2'-dimethyl-; 5,5'-dimethyl; 6,6'-dimethyl-; and 7,7'-dimethylDIM; and 1,1',2,2'-tetramethylDIM. The six compounds exhibited minimal to non-detectable AhR agonist or antagonist activities associated with CYP1A1 induction. In contrast, the methyl-substituted DIMs inhibited estrogen-induced T47D human breast cancer cell growth. The antitumorigenic activity of these compounds was examined in 7,12-dimethylbenz[a]anthracene-induced rat mammary tumor model in which the DIM analogs were orally administered (by gavage in corn oil) at a dose of 1 mg/kg/every second day (X10). 1,1'-DimethylDIM, 5,5'-dimethylDIM and 1,1',2,2'-tetramethylDIM significantly inhibited mammary tumor growth, and this was not accompanied by changes in organ/body weights or histopathology. These studies demonstrate that methyl-substituted DIMs are selective AhR modulators (SAhRMs) with potential for clinical treatment of breast cancer.

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Introduction

Research in our laboratory has been focused on the mechanism of inhibitory aryl hydrocarbon receptor (AhR)-estrogen receptor α (ERα) crosstalk in breast cancer cells and results indicate that AhR agonists inhibit estrogen (E2)-induced gene expression and cell proliferation (1,2). Moreover, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a high affinity ligand for the AhR, inhibits age-dependent and carcinogen-induced mammary tumor formation and growth in female Sprague-Dawley rats, and a recent study reported that women accidentally exposed to TCDD in Seveso, Italy, over 20 years ago exhibit lower incidence rates of breast and endometrial cancer (3). Studies on various structural classes of AhR agonists have identified alternate substituted (1,3,6,8- or 2,4,6,8-) alkyl polychlorinated dibenzofurans (PCDFs) and substituted diindolylmethanes (DIMs) as selective AhR modulators (SAhRMs) that are relative non-toxic but inhibit mammary tumor growth in rodent models (4). With financial support from this grant, I have been investigating the indirect antiestrogenic activity of substituted DIMs and applications of these compounds for treating mammary cancer (5-7).

Body

Uterine responses and AhR activation

Initial studies described in the previous Annual Summary Report showed that a series of methyl-substituted DIMs (Fig. 1) inhibited growth of 7,12-dimethylbenz[a]anthracene-induced mammary tumors in female Sprague-Dawley rats, and these compounds also inhibited E2-induced proliferation of estrogen receptor (ER)-positive T47D breast cancer cells. The antitumorigenic potency of some of these compounds, such as 2,2'-dimethylDIM, was as low as 1 mg/kg/2 days (<0.5 mg/kg/day), and this was not accompanied by changes in organ/tissue histology, whole body or organ weights (liver, uterus, heart, spleen, and kidney). The antiestrogenic activity of the most active methyl-substituted DIMs (i.e. 1,1'-dimethyl-; 2,2'-dimethyl-, 5,5'-dimethyl-; and 1,1',2,2'-tetramethylDIM) was also investigated in the immature female B6C3/F1 mice in which E2 (0.05 μg/mouse), test compound (100 mg/kg/day) or their combination was administered every 24 h for 3 days, and mice were then sacrificed 24 h after the final treatment. The effects of the various compounds on uterine wet weight, progesterone receptor (PR) binding, peroxidase activity, and CYP1A1-dependent ethoxyresorufin *O*-deethylase (EROD) were determined (Tables 1 and 2). The results show that for several E2-induced responses, treatment with E2 induced a >3-, 16- and 7-fold increase in uterine wet weight, PR binding and uterine peroxidase activity, respectively, as previously described. The only induction responses observed for the methyl-substituted DIMs alone was a < 3-fold induction of uterine PR binding by 2,2'-diMeDIM and 1,1',2,2'-tetraMeDIM. In animals cotreated with methyl-substituted DIMs plus E2 (Table 2), all compounds inhibited E2-induced PR levels and with the exception

of 1,1',2,2'-tetraMeDIM, inhibition of uterine peroxidase activity was also observed. Only 1,1'-diMeDIM inhibited E2-induced uterine wet weight increase. These data show that methyl-substituted DIMs inhibit one or more E2-induced mouse uterine responses, and these data are comparable to results previously reported for DIM and dihaloDIMs (5, 6).

Previous studies (5-7) showed that SAhRMs and other AhR agonists inhibit E2-induced responses in the rodent uterus, and some inhibitory effects were also observed for four of the more active (*in vitro*) methyl-substituted DIMs at a dose of 100 mg/kg/day (X3) (Tables 1 and 2). The rodent uterus is generally the least sensitive organ for observing inhibitory AhR-ER crosstalk, and this was consistent with results obtained for compounds investigated in this study and for the dihaloDIMs (6). Interestingly, the high dose antiestrogenic responses observed for DIM and related compounds in the rodent uterus (300 mg/kg - total dose) was not accompanied by induction of hepatic CYP1A1-dependent EROD activity which is usually induced in concert with AhR-mediated toxic responses in the rodent. The lack of CYP1A1 induction was also observed in breast cancer cell lines, and results of hepatic cytosolic transformation assay (Fig. 2) indicated that the methyl substituted DIMs interacted with the AhR complex. For example, 5 nM TCDD (positive control) and 5 μM DIM analogs transformed the receptor, indicating that the DIMs bound the AhR. In contrast, lower doses exhibited minimal transforming activity, indicating that the methyl-substituted DIMs were ≥ 1000 times less active than TCDD in this assay. These results suggest that the high antitumorigenic activity for some of the methyl-substituted DIMs (e.g. < 500 μg/kg/day) may be due to inhibitory AhR-ERα crosstalk. It is also possible that the high anticarcinogenic activity of the

methyl-substituted DIMs may also be due to activation of other inhibitory pathways, and these are currently being investigated.

Training

I have been completing the fellowship originally awarded to Ms. Mona Sethi-Gupta and have contributed to the ongoing studies on substituted DIMs. Results of my studies were presented at the Society of Toxicology national meeting in San Francisco, CA (March, 2001) and at the Gulf Coast Society of Toxicology meeting in Austin, TX (November, 2000). In addition, I attended the 20^{th} International Symposium on Halogenated Environmental Organic Pollutants and POPs (August, 2000; Monterey, CA) and the Gordon Conference on Hormone Action (August 2001, Lebanon, NH).

Key Research Accomplishments

- Alkylated DIMs exhibit antiestrogenic activity in the mouse uterus at high doses (100 mg/kg/day X3).

- Like other SAhRMs, alkylated DIMs did not induced hepatic CYP1A1-dependent EROD activity.

- Alkyl DIMs did not induce EROD activity on T47D breast cancer cells or reporter gene activity (CAT) in cells transfected with an Ah-responsive construct; however, these compounds transformed the AhR complex at high concentrations (5 μM).

- The results suggest that alkyl DIMs may be active through AhR-dependent and -independent pathways.

Reportable Outcomes

(a) Manuscripts, abstracts, presentations

McDougal, A., Gupta, M.S., Morrow, D., Ramamoorthy, K., Lee, Y.-E., and Safe, S. Methyl-substituted diindolylmethanes as inhibitors of estrogen-induced growth of T47D cells and mammary tumors in rats. *Breast Cancer Res. Treat.* 66:147-157, 2001.

McDougal, A.; Gupta, M.S., Morrow, D., Ramamoorthy, K., Lee, J. and Safe, S. Methyl-substituted diindolylmethanes as inhibitors of estrogen-induced growth of T47D cells and mammary tumors in rats. *Toxicologist* 55: 1413, 2001.

Safe, S., McDougal, A., Gupta, M.S., and Ramamoorthy, K. Selective Ah receptor modulators (SAhRMs): progress towards development of a new class of inhibitors of breast cancer growth. *J. Women's Cancer* in press, 2001.

(b) Patents/ licenses applied for or issued

none

(c) Degrees

Mona Sethi-Gupta - Ph.D. (2000)
"Mechanistic Studies of Xenobiotic and Natural Compounds that Modulate Estrogen Receptor and Aryl Hydrocarbon Receptor Signaling Pathways"

(d) Cell lines/serum

No new lines developed.

(e) Informatics

None

(f) Funding applied for

None

(g) Employment/research opportunities

Mona Sethi-Gupta - Postdoctoral Fellowship, Medical College of Virginia, Richmond VA

Conclusions

The results obtained for halo- and methyl-substituted DIMs demonstrate that some of these compounds are highly active as inhibitors of mammary tumor growth and agonists for the Ah receptor. The potency of these compounds suggests that they may act through more than one pathway or receptor, and I will investigate the possibility that substituted DIMs bind other ligand-activated nuclear receptors. This may lead to new anticancer agents for treatment of breast cancer which activate multiple inhibitory pathways.

References

1. Safe S: 2,3,7,8-Tetrachlorodibenzo-*p* -dioxin (TCDD) and related environmental antiestrogens: characterization and mechanism of action. In: Naz RK (ed.) Endocrine Disruptors. CRC Press, Boca Raton, FL, 1999, pp. 187-221

2. Zacharewski T, Safe S: Antiestrogenic activity of TCDD and related compounds. In: Korach KS (ed.) Reproductive and Developmental Toxicology. Marcel Dekker, Inc., New York, 1998, pp. 431-448

3. Bertazzi, P.A., Consonni, D., Bachetti, S., Rubagotti, M., Baccarelli, A., Zocchetti, C., and Pesatori, A.C. Health effects of dioxin exposure: A 20-year mortality study. *Am. J. Epidemiol.* 153:1031-1044, 2001.

4. Safe S, Qin C, McDougal A: Development of selective aryl hydrocarbon receptor modulators (SARMs) for treatment of breast cancer. Expert Opinion on Investigational Drugs 8: 1385-1396, 1999

5. Chen I, McDougal A, Wang F, Safe S: Aryl hydrocarbon receptor-mediated antiestrogenic and antitumorigenic activity of diindolylmethane. Carcinogenesis 19: 1631-1639, 1998

6. McDougal A, Sethi-Gupta M, Ramamoorthy K, Sun G, Safe S: Inhibition of carcinogen-induced rat mammary tumor growth and other estrogen-dependent responses by symmetrical dihalo-substituted analogs of diindolylmethane. Cancer Letts. 151: 169-179, 2000

7. McDougal, A., Gupta, M.S., Morrow, D., Ramamoorthy, K., Lee, Y.-E., and Safe, S. Methyl-substituted diindolylmethanes as inhibitors of estrogen-induced growth

of T47D cells and mammary tumors in rats. *Breast Cancer Res. Treat.* 66:147-157, 2001.

Table 1. Estrogenic activity of alkyl-substituted DIMs in the immature mouse uterus.

Compound	Uterine Wet Weight (mg) (% CO act)	PR (fmol/uterus) (% CO act)	UPO (units/mg) (% CO act)	EROD (pmol/min/mg)
Corn oil (vehicle)	8.0 ± 0.94 (100)	75 ± 46 (100)	0.11 ± 0.01 (100)	13.9 ± 1.4
E2 (0.02 μg/mouse)	25.4 ± 9.8* (318)	1208 ± 59* (1161)	0.78 ± 0.05* (709)	13.1 ± 2.1
1,1'-diMeDIM (100 mg/kg)	8.3 ± 1.8 (104)	33 ± 11 (44)	0.13 ± 0.01 (118)	14.9 ± 1.1
2,2'-diMeDIM (100 mg/kg)	9.9 ± 2.4 (124)	215 ± 57* (287)	0.15 ± 0.02 (136)	12.6 ± 0.3
5,5'-diMeDIM (100 mg/kg)	9.1 ± 0.75 (114)	54 ± 17 (72)	0.15 ± 0.01 (136)	16.5 ± 0.3
1,1',2,2'-tetraMeDIM (100 mg/kg)	9.5 ± 1.2 (119)	158 ± 44* (211)	0.11 ± 0.01 (100)	12.9 ± 0

Mice were treated with corn oil (control), E2 or the methyl-substituted DIMs (100 mg/kg) for three successive days and uterine wet weight, PR binding activity (UPO), peroxidase activity, and EROD activity were determined as described in the Materials and Methods. * Significantly different from control group ($p < 0.05$). Statistical significance was determined using Dunnett's one-tailed test (ANOVA).

Table 2. Effects of E2 and E2 plus methyl-substituted DIMs on uterine activities in immature female B6C3F1 mice.

Compound	Uterine wet weight (mg) (% E2 act)	PR (fmol/uterus) (% E2 act)	UPO (units/mg) (% E2 act)
Corn oil	11.9 ± 1.8	533 ± 153	0.19 ± 0.01
E2 (0.02 μg/mouse)	41.0 ± 5.2	2186 ± 188	2.57 ± 0.01
E2 + 1,1'-diMeDIM	36.9 ± 3.4 (90)*	1724 ± 212 (79)*	1.77 ± 0.02 (69)*
Corn oil	12.1 ± 1.7	350 ± 47	0.19 ± 0.01
E2 (0.02 μg/mouse)	30.1 ± 3.3	1568 ± 100	1.14 ± 0.04
E2 + 2,2'-diMeDIM	29.1 ± 2.3 (97)	1044 ± 38 (67)*	0.98 ± 0.01 (86)*
Corn oil	11.3 ± 2.6	218 ± 36	0.14 ± 0.02
E2 (0.02 μg/mouse)	36.2 ± 7.6	1520 ± 91	1.38 ± 0.01
E2 + 5,5'-diMeDIM	35.2 ± 4.5 (97)	1118 ± 59 (74)*	0.79 ± 0.04 (86)*
Corn oil	13.4 ± 3.5	111 ± 282	0.17 ± 0.00
E2 (0.02 μg/mouse)	31.6 ± 6.1	1356 ± 519	1.07 ± 0.8
E2 + 1,1',2,2'-tetrameDIM	31.8 ± 6.7 (101)	608 ± 387 (45)*	1.26 ± 0.01 (118)

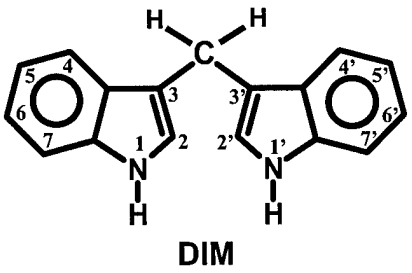
Mice were treated with corn oil (control), E2 alone or in combination with the methyl-substituted DIMs for three successive days and uterine wet weight, PR binding activity, and peroxidase activity (UPO) were determined as described in the Materials and Methods.

* Cotreatments that are significantly different from E2-treated groups ($p < 0.05$). Statistical significance was determined using Dunnett's one-tailed test (ANOVA).

Cotreatments = E2 + 100 mg/kg substituted DIM.

This Table summarizes results from four separate experiments.

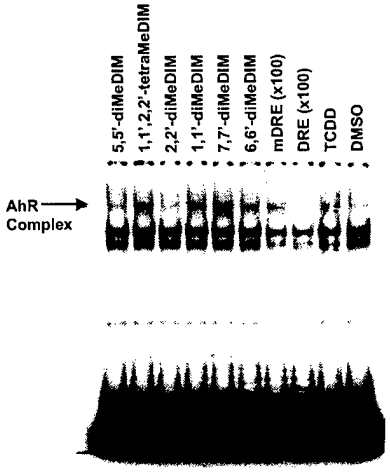
Figure 1. Structure of methyl-substituted DIMs.



DIM

1,1'-diMeDIM 2,2'-diMeDIM 1,1',2,2'-tetraMeDIM
5,5'-diMeDIM, 6,6'-diMeDIM, 7,7'-diMeDIM

Figure 2. Interactions of methyl-substituted DIMs with the AhR in gel mobility shift assays. Rat hepatic cytosol was incubated with 5 μM concentrations of methyl-substituted DIMs, 5 nM TCDD in DMSO, or DMSO alone, and formation of the specifically bound retarded band was determined in gel mobility shift assays as described in the Materials and Methods. Intensity of the retarded band was decreased after competition with 100-fold excess of wild-type (lane 8), but not mutant (lane 7) DRE oligonucleotides. In duplicate experiments, all DIM compounds transformed the AhR; however, there was some variability in band intensities between studies.



Breast Cancer Research and Treatment **66:** 147–157, 2001.

Report

Methyl-substituted diindolylmethanes as inhibitors of estrogen-induced growth of T47D cells and mammary tumors in rats

Andrew McDougal, Mona Sethi Gupta, Derek Morrow, Kavita Ramamoorthy, Jeong-Eun Lee, and Stephen H. Safe

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Key words: antitumorigenic, selective AhR modulators, substituted diindolylmethanes

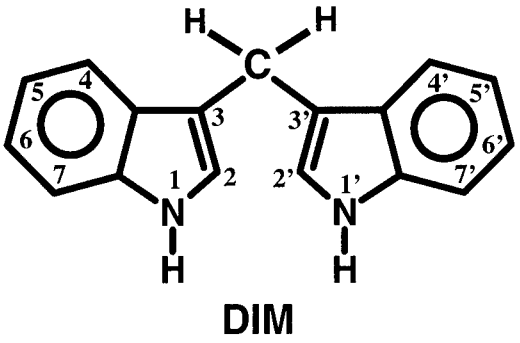
Summary

Diindolylmethane (DIM) is formed by acid catalyzed dimerization of the phytochemical indole-3-carbinol, and both compounds inhibit formation and/or growth of mammary tumors in rodents. In this study, we have investigated the aryl hydrocarbon receptor (AhR) agonist activity and inhibitory AhR-estrogen receptor crosstalk induced by the following methyl-substituted DIMs: 1,1′-dimethyl-, 2,2′-dimethyl-, 5,5′-dimethyl-, 6,6′-dimethyl-, and 7,7′-dimethylDIM and 1,1′,2,2′-tetramethylDIM. The six compounds bound to the rat cytosolic AhR in a transformation assay but, at concentrations $\leq 10\,\mu M$, exhibited minimal to non-detectable AhR agonist or antagonist activities associated with CYP1A1 induction. In contrast, the methyl-substituted DIMs inhibited estrogen-induced T47D human breast cancer cell growth and the four most active compounds (1,1′-, 2,2′-, 5,5′-dimethylDIM and 1,1′,2,2′-tetramethylDIM) inhibited one or more estrogen-induced responses in the 21-day-old female B6C3F1 mice at a dose of 100 mg/kg/day (X3). Induction of hepatic CYP1A1-dependent activity was not observed at this high dose. The antitumorigenic activity of these compounds was examined in 7,12-dimethylbenz[a]anthracene-induced rat mammary tumor model in which the DIM analogs were orally administered (by gavage in corn oil) at a dose of 1 mg/kg/day (X10). 1,1′-DimethylDIM, 5,5′-dimethylDIM and 1,1′,2,2′-tetramethylDIM significantly inhibited mammary tumor growth, and this was not accompanied by changes in organ/body weights or histopathology. These studies demonstrate that methyl-substituted DIMs are selective AhR modulators (SAhRMs) with potential for clinical treatment of breast cancer.

Introduction

Breast cancer is one of the leading causes of premature death in women and it is estimated that one in eight women in the United States will be diagnosed with this disease [1–3]. Most early stage mammary tumors are estrogen receptor (ER)-positive and are responsive to endocrine therapy which includes treatment with drugs such as tamoxifen that bind the ER and exhibit antiestrogenic and antitumorigenic activity in the breast [4]. Both steroidal and nonsteroidal selective ER modulators (SERMs) are being developed for treatment of breast cancer and other estrogen-related problems, and SERMs typically exhibit tissue-dependent ER antagonist, agonist or mixed agonist/antagonist activities [5–7]. Various ligands for the retinoic acid, vitamin D, peroxisome proliferator activated, and aryl hydrocarbon (Ah) receptors are also being developed for treatment of breast cancer due to their crosstalk with the ER and inhibition of both 17β-estradiol (E2)-induced gene expression and mammary tumor growth [8–15].

The Ah receptor (AhR) is a ligand-activated basic helix-loop-helix transcription factor that forms a nuclear heterodimeric complex with the AhR nuclear translocator (Arnt) protein [16–20]. The nuclear AhR complex activates transcription of target genes such as CYP1A1 by interacting with dioxin response elements (DREs) in promoter regions of Ah responsive genes [19–21]. The AhR binds structurally diverse



1,1'-diMeDIM 2,2'-diMeDIM 1,1',2,2'-tetraMeDIM
5,5'-diMeDIM, 6,6'-diMeDIM, 7,7'-diMeDIM

Figure 1. Structure of methyl-substituted DIMs.

ligands that include the toxic environmental contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related halogenated aromatics, as well as naturally-occurring compounds including phytochemicals, such as carotenoids, flavonoids, indole-3-carbinol (I3C) and related heteroaromatics including diindolylmethane (DIM) [13, 22–29].

TCDD induces a broad spectrum of toxic response and modulates several endocrine signaling pathways [19]. For example, TCDD is a potent antiestrogen and inhibits E2-induced gene expression and growth in the rodent uterus, human breast cancer cells, and rodent mammary tumors [reviewed in 12–15]. Research in our laboratory has focused on delineating the mechanisms of inhibitory AhR-ER crosstalk and development of relatively nontoxic selective AhR modulators (SAhRMs) for treatment of breast cancer [13]. Previous studies show that alternate substituted alkyl polychlorinated dibenzofurans (PCDFs), DIM, and halogen-substituted DIMs are relatively nontoxic SAhRMs that inhibit mammary tumor growth in carcinogen-induced female Sprague-Dawley rats [30–32]. This study also shows that methyl-substituted DIMs (Figure 1) also inhibit E2-induced growth of human breast cancer cells and carcinogen-induced rodent mammary tumors, and thereby represent the first examples of synthetic non-chlorinated SAhRMs that can be used for AhR-based treatment of breast cancer.

Materials and methods

Chemicals and biochemicals

The 1-methyl, 2-methyl, 1,2-dimethyl, 5-methyl, 6-methyl and 7-methylindoles were all purchased from Aldrich Chemical Co. (Milwaukee, WI). The corresponding symmetrical di- and tetramethyl DIMs were prepared by conversion of the substituted indoles into the corresponding carboxaldehydes by condensation with dimethylformamide; sodium borohydride was used to reduce the carboxaldehydes into the light sensitive methyl-substituted indole-3-carbinols which were dimerized by stirring in aqueous buffer for 24–48 h as described [32]. The resulting crude condensation products were recovered by filtration and crystallized from benzene/hexane to give 95–99% pure products as determined by gas chromatrography-mass spectrometry. Nuclear magnetic resonance spectra (400 MHz) were obtained in deuterochloroform to give the following: 1,1′-diMeDIM, 3.72 (CH_3, s), 6.80 (H2, s), 7.09 (H5, t), 7.22 (H6, t), 7.29 (H7, d) and 7.63 (h4,d); 2,2′-diMeDIM, 2.33 (CH_3, s), 6.96 (H6, t), 7.06 (H5, t), 7.22 (H7, d), 7.40 (H4, d) and 7.68 (H1, s); 1,1′,2,2′-tetraMeDIM, 2.38 and 3.65 (CH_3, s), 6.96 (H6, t), 7.06 (H5, t), 7.22 (H7 d), 7.42 (H4, d); 5.5′-diMeDIM, 2.45 (CH_3, s), 6.87 (H2, s), 7.03 (H6, d), 7.25 (H7, d), 7.43 (H4, s), 7.74 (H1, s); 6,6′-diMeDIM, 6.82 (H2, s), 6.92 (H5, d), 7.12 (H7, s), 7.49 (H4, d) and 7.72 (H1, s); 7.7′-diMeDIM, 6.90 (H1, s), 7.02 (H6, t), 7.04 (H5, d), 7.50 (H4, s) and 7.75 ppm (H1, s). Ortho-coupled protons gave doublets (d) with coupling constants of 8.0–8.4 Hz; protons with two ortho substituents gave triplets (t) with coupling constants of 8.0–8.4 Hz and the H1 resonance was a broad single (s). T47D breast cancer cells were obtained from the American Type Culture Collection (Manassas, VA); α-minimum essential medium (αMEM) and fetal bovine serum (FBS) were purchased from Life Technologies (Grand Island, NY) and Intergen (Purchase, NY), respectively. All other biochemials were the highest quality available from commercial sources and TCDD (> 98%) was prepared in this laboratory. The wild-type and mutant dioxin responsive element (DRE) oligonucleotides were synthesized by the DNA Technologies Laboratory, Texas A&M University (College Station, TX).

wild-type DRE: 5′-GAT CTC CGG TCC TTC TCA CGC AAC GCC TGG GG-3′

mutant DRE: 5′-GAT CTC CGG TCC TTC Tac atC AAC GCC TGG GG-3′

The Ah-responsive pRNH11c construct contains the regulatory human CYP1A1 region from the Taq I site at −1142 to the BcII site at +2434 fused to the bacterial chloramphenicol acetyltransferase (CAT) reporter gene (kindly provided by Dr. R. Hines, Medical College of Wisconsin, Milwaukee, WI)

Uterine assays in B63F1 mice

Twenty-one-day-old B6C3F1 female mice were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were dosed every 24 h on days 21, 22, and 23 of age with corn oil (control) by gavage, 0.02 μg/day E2 by i.p. injection, or 0.02 μg/day E2 by i.p. injection plus 100 mg/kg of substituted DIMs by gavage. Animals were sacrificed 20 h after the last treatment. Uterine wet weights, cytosolic PR binding activity and peroxidase activity were determined as previously described [32].

Mammary tumor studies

Virgin female Sprague-Dawley rats were obtained from Harlan (Houston, TX) and at 50 days of age were treated orally with a single dose of DMBA (20 mg/rat). Within 45–75 days, tumors could be detected by palpation; when the tumors reached a small predetermined size (100–200 mm^3), rats were treated by gavage with corn oil (vehicle) or test compounds dissolved in corn oil every second day for 20 days at a final volume of 2 ml/kg, with 8–10 rats per treatment group [30–32]. Tumors were measured with calipers and volume calculated by the formula: (length/2) × (width/2) × (length/2) × π. Rats treated with 1,1′-diMeDIM, 2,2′-diMeDIM and 1,1′,2,2′-MeDIM were euthanized on day 21; rats treated with 5,5′-diMeDIM were euthanized on day 19. Tumors and selected organs were excised, weighed, and processed for histopathological examination. Hepatic microsomal extracts were prepared and analyzed for ethoxyresorufin *O*-deethylase (EROD) activity in triplicate using a plate reader (Millipore, Watertown, MA) as previously described [30–32].

T47D cell proliferation assay

T47D human breast cancer cells were maintained in αMEM supplemented with 5% FBS, sodium bicarbonate (2.2 g/l) and gentamycin/penicillin/streptomycin (10,000 units/l; 10 mg/l). T47D cells were seeded into 6-well plates (50,000/well) in DME-F12 without phenol red supplemented with 5% dextran-coated charcoal-treated FBS, sodium bicarbonate and antibiotic/antimycotic solution. After 24 h, cells were treated with 1 nM E2 alone, substituted DIMs, or 1 nM E2 + DIMs in DMSO, with DMSO (vehicle control) at a final concentration of 0.2% (v/v) in all wells. Media was changed every 48 h, and cells were again treated with the same concentrations of chemicals/hormones. Four different concentrations of DIM (0.1, 1.0, 5 and 10 μM) were used in this study, and the compounds alone did not significantly affect growth of T47D cells or exhibit cytotoxicity at these concentrations. At higher concentrations, cell proliferation was inhibited and floating dead cells were observed. After 14 days, cells were harvested and counted using a Coulter Z1 cell counter (Coulter Electronics, Hialeah, FL).

Gel electromobility shift assay

Nine pmol synthetic human dioxin responsive element (DRE) oligonucleotide was 5′-end labeled with T4-polynucleotide kinase and [A-^{32}P]ATP and incubated with 5 μl 10X phosphorylation buffer, 3 μl polynucleotide kinase (10 μ/μl), 33 μl H_2O at 37°C for 30 min. The mixture was purified through a TE-10 column. Rat hepatic cytosol was prepared and incubated (80 μg/reaction) with DMSO or substituted DIMs in DMSO, final concentration 0.1% (vol/vol) at 20°C for 2 h, and then with 1 μg poly[d(I-C)] at 20°C for 15 min. For competition experiments, a 100-fold excess of unlabeled wild type or mutant DRE oligonucleotide was added. [^{32}P]-labeled DRE oligonucleotide DNA was then added and incubated for an additional 15 min at 20°C. Reaction mixtures were loaded onto a 5% polyacrylamide gel and electrophoresed at 120V in Tris buffer (0.9 M Tris-borate, 2 mM EDTA, pH 8.0) for 2.5 h. Gels were dried and visualized by autoradiography using a Packard Instant Imager (Downers Grove, IL).

Statistics

Six to eight mice and 8–10 rats were used in each treatment group. Cell proliferation assays were performed in triplicate, and repeated on a separate occasion at least once. Data was analyzed for significant differences using ANOVA and Duncan's New Multiple Range, and results are expressed as means ± SE or SD for each treatment group.

Results

Cell proliferation studies

Initial screening of the methyl-substituted DIMs used cell proliferation assays in T47D human breast cancer cells that are responsive to the mitogenic effects of E2 but are also inhibited by cotreatment with AhR agonists [32]. The results illustrated in Figure 2 summarize

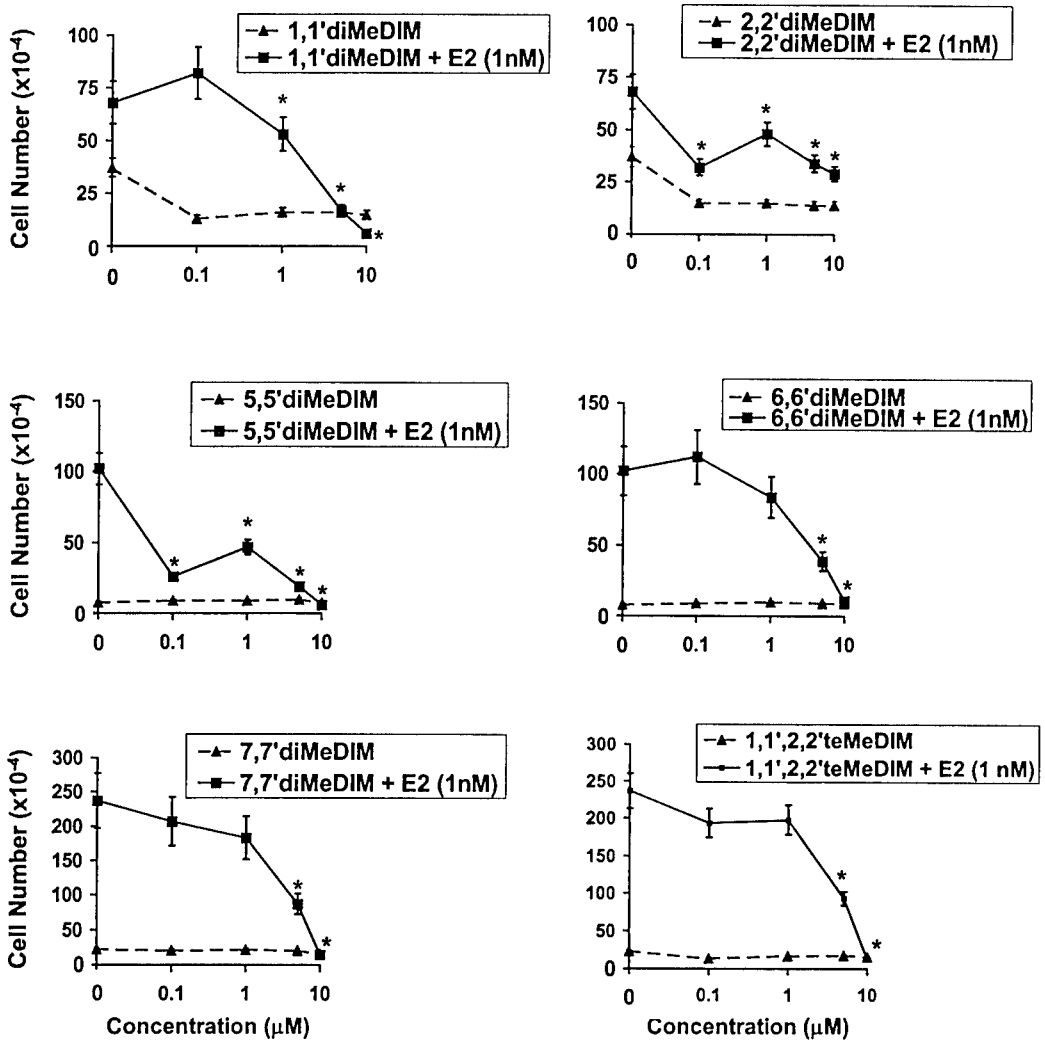


Figure 2. Estrogenic and antiestrogenic activities of methyl-substituted DIMs in T47D cells. Effects of methyl-substituted DIMs (100 nM–10 μM) alone or in combination with 1 nM E2 on T47D cell proliferation were determined. Results are the average of three replicate experiments expressed ± SE. *Significant inhibition ($p < 0.05$) of E2-induced cell proliferation.

effects of six methyl-substituted DIMs alone and in combination with E2 on growth of T47D cells. The number of cells in the vehicle-treated (DMSO) group is shown where the dashed line intersects the *y*-axis. A 2- to 6-fold increase in cell numbers was observed in cells treated with DMSO alone. The compounds alone had minimal effects on growth of the cells (compared to DMSO) using concentrations of 0.1, 1.0, 5.0 and 10 μM. In cotreatment studies, 5 and 10 μM concentrations of all methyl-substituted DIMs inhibited E2-induced proliferation of T47D cells and the most active compound significantly inhibited growth at concentrations of 0.1 (2,2′-diMeDIM and 5,5′-diMeDIM) or 1.0 μM (1,1′-diMeDIM). The results indicate that methyl-substituted DIMs exhibit antiestrogenic activity and this included compounds substituted at the hetero N atom, and in both rings of the indole moiety.

Methyl-substituted DIMs as AhR agonists

Interaction of methyl-substituted DIMs with the AhR was determined by transformation of rat hepatic cytosolic AhR to a DNA binding form that can be readily detected in a gel mobility shift assay (Figure 3). The results showed that 5 nM TCDD (positive control) and 5 μM DIM analogs transformed the receptor, indicating that the DIMs bound the AhR. In contrast, lower doses exhibited minimal transform-

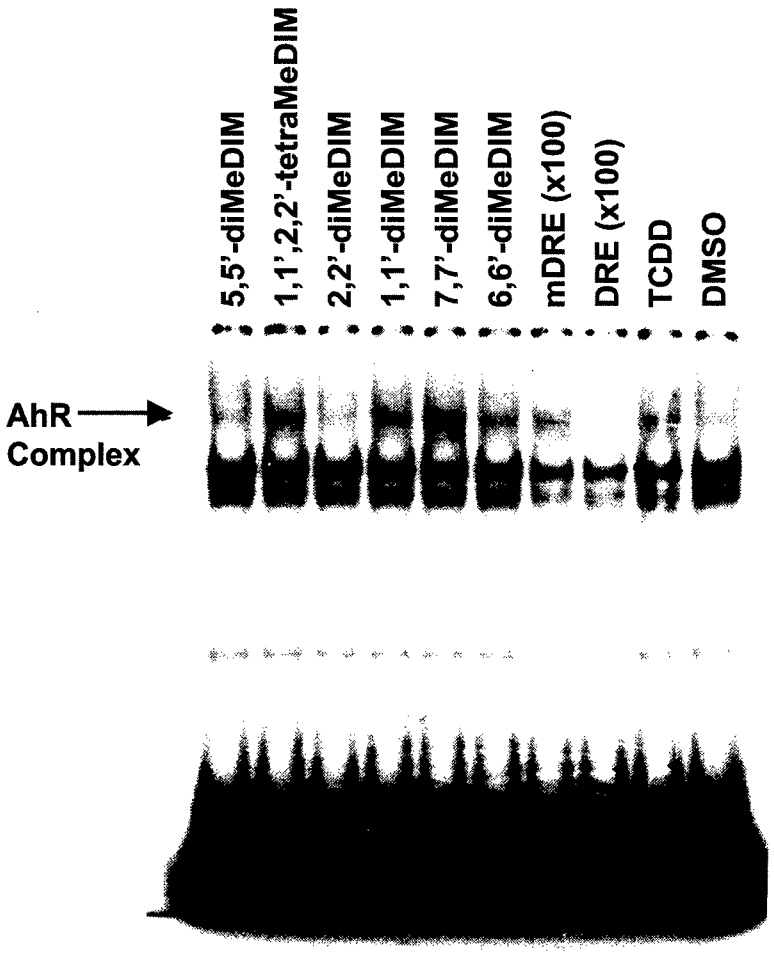


Figure 3. Interactions of methyl-substituted DIMs with the AhR in gel mobility shift assays. Rat hepatic cytosol was incubated with 5 μM concentrations of methyl-substituted DIMs, 5 nM TCDD in DMSO, or DMSO alone, and formation of the specifically bound retarded band was determined in gel mobility shift assays as described in the Materials and methods. Intensity of the retarded band was decreased after competition with 100-fold excess of wild-type (lane 8), but not mutant (lane 7) DRE oligonucleotides. In duplicate experiments, all DIM compounds transformed the AhR; however, there was some variability in band intensities between studies.

ing activity, indicating that the methyl-substituted DIMs were ≥ 1000 times less active than TCDD in this assay. Initial studies showed that all six methyl-substituted DIMs did not induce CYP1A1-dependent EROD activity in T47D human breast cancer cells, but in cells cotreated with these compounds plus DIM, there was complete inhibition of induced EROD activity (data not shown). The apparent AhR antagonist activity of the dimethylDIMs in this assay is ambiguous since these compounds directly interact with CYP1A1 and are potent inhibitors of CYP1A1-dependent activity [23]. The AhR agonist/antagonist activity of these compounds was therefore investigated in T47D cells transfected with Ah-responsive pRNH11c containing the -1142 to $+2434$ region of the human CYP1A1 gene promoter fused to the bacterial CAT reporter gene (Figure 4). The results show that at concentrations as high as 10 μM, the substituted DIMs did not induce CAT activity and in cells cotreated with TCDD plus tetra-diMeDIMs, CAT activity induced by TCDD was not inhibited. These results indicate that 10 μM concentrations of the methyl-substituted DIMs exhibited minimal AhR agonist or antagonist activity associated with induction of CYP1A1, whereas these same compounds were active as antiestrogens in this cell lines (Figure 2). Similar differential responsiveness has previously been observed for other SAhRMs including alternate substituted alkyl PCDFs, DIM and dihaloDIMs [30–32].

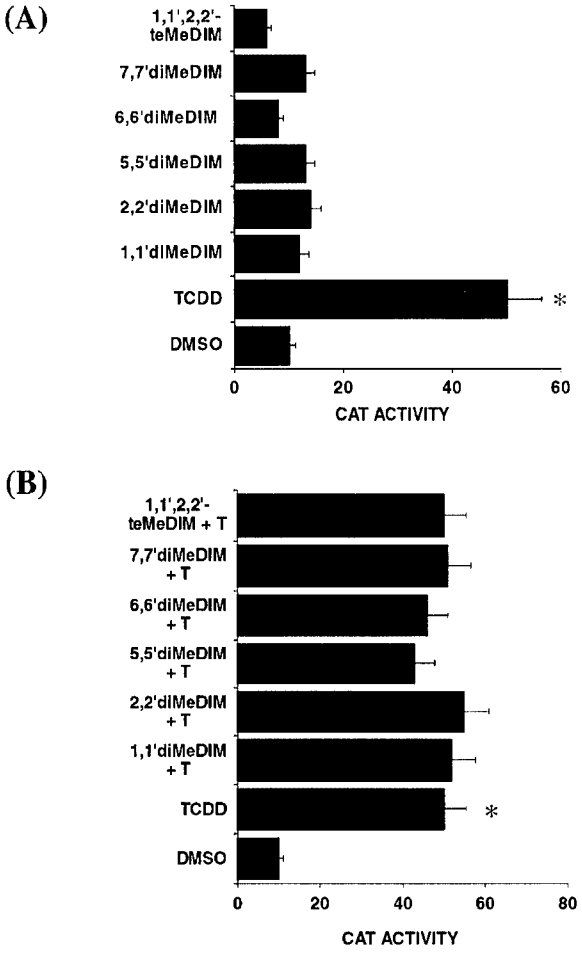


Figure 4. AhR agonist (A) and antagonist (B) activities of methyl-substituted DIMs in T47D cells transfected with pRNH11c. Cells were treated with DMSO (control), 1 nM TCDD (T), and 10 μM DIM compounds alone or in combination with TCDD. CAT activities were determined. Results are expressed as means ± SE for three separate experiments for each treatment group. *Significant induction ($p < 0.05$) of CAT activity. TCDD induced CAT activity, but the DIM compounds did not exhibit AhR agonist or antagonist activities for this response.

In vivo *antiestrogenic activities of tetra-DiMeDIMs*

Based on results of initial *in vitro* studies described above, four of the more active compounds were selected for further *in vivo* studies in the mouse uterus: 1,1′-diMeDIM; 2,2′-diMeDIM; 5,5′-diMeDIM and 1,1′,2,2′-tetraMeDIM. The effects of these compounds alone (100 mg/kg) and in combination with E2 (0.02 μg/mouse) were determined in immature 21-day-old female B6C3F1 mice where compounds were administered on three consecutive days and sacrificed 24 h after the third treatment (Tables 1 and 2). The results show that E2 induced a > 3-, 16- and 7-fold increase in uterine wet weight, PR binding and uterine peroxidase activity, respectively. The only induction responses observed for the methyl-substituted DIMs was a < 3-fold induction of uterine PR binding by 2,2′-diMeDIM and 1,1′,2,2′-tetraMeDIM. In animals cotreated with methyl-substituted DIMs plus E2 (Table 2), all compounds inhibited E2-induced PR levels and with the exception of 1,1′,2,2′-tetraMeDIM, inhibition of uterine peroxidase activity was also observed. Only 1,1′-diMeDIM inhibited E2-induced uterine wet weight increase. These data show that methyl-substituted DIMs inhibit one or more E2-induced mouse uterine responses, and these data are comparable to results previously reported for DIM and dihaloDIMs [31, 32].

The results in Figure 5 summarize the antitumorigenic activity of the four methyl-substituted DIMs using the DMBA-induced rat mammary tumor model [30–32]. Fifty day-old female Sprague-Dawley rats were initiated with DMBA, and within 6–12 weeks, mammary tumors developed. Animals were then treated with corn oil (control) or DIM analogs in corn oil every other day for 20 days and sacrificed on day 21. Initial studies showed that 1,1′-diMeDIM was active at a dose of 5 mg/kg (Figure 5A) and subsequent experiments used a dose of 1 mg/kg. The results show that 5,5′-diMeDIM, 2,2′-diMeDIM and 1,1′,2,2′-tetraMeDIM significantly inhibited tumor growth (Figures 5B and 5C, Table 3) but did not alter body or organ weights or induce hepatic microsomal EROD activity. In contrast, 1,1′-diMeDIM did not significantly inhibit tumor growth at a dose of 1 mg/kg, and 7,7′-DIM was also inactive at this dose (data not shown). Treatment with methyl substituted DIMs did not significantly affect tumor number; however, in this animal model, tumor multiplicity was < 3 in most animals. Thus, three of the methyl-substituted DIMs were antitumorigenic at the 1.0 mg/kg dose and were more active than DIM which inhibited tumor growth at the 5 mg/kg but not 1 mg/kg dose level [31].

Discussion

The AhR is a ligand-activated transcription factor that modulates multiple biochemical pathways in a tissue-, age-, sex- and species-dependent manner. Inhibitory AhR-ER crosstalk in the rodent uterus and mammary tumors and in human breast cancer cells has been extensively characterized [12–15], and two different structural classes of SAhRMs have been investigated

Table 1. Estrogenic activity of alkyl-substituted DIMs in the immature mouse uterus

Compound	Uterine Wet Weight (mg) (% CO act)	PR (fmol/uterus) (% CO act)	UPO (units/mg) (% CO act)	EROD (pmol/min/mg)
Corn oil (vehicle)	8.0 ± 0.94 (100)	75 ± 46 (100)	0.11 ± 0.01 (100)	13.9 ± 1.4
E2 (0.02 μg/mouse)	25.4 ± 9.8* (318)	1208 ± 59* (1161)	0.78 ± 0.05* (709)	13.1 ± 2.1
1,1′-diMeDIM (100 mg/kg)	8.3 ± 1.8 (104)	33 ± 11 (44)	0.13 ± 0.01 (118)	14.9 ± 1.1
2,2′-diMeDIM (100 mg/kg)	9.9 ± 2.4 (124)	215 ± 57* (287)	0.15 ± 0.02 (136)	12.6 ± 0.3
5,5′-diMeDIM (100 mg/kg)	9.1 ± 0.75 (114)	54 ± 17 (72)	0.15 ± 0.01 (136)	16.5 ± 0.3
1,1′,2,2′-tetraMeDIM (100 mg/kg)	9.5 ± 1.2 (119)	158 ± 44* (211)	0.11 ± 0.01 (100)	12.9 ± 0

Mice were treated with corn oil (control), E2 or the methyl-substituted DIMs (100 mg/kg) for three successive days and uterine wet weight, PR binding activity (UPO), peroxidase activity, and EROD activity were determined as described in the Materials and methods.
*Significantly different from control group ($p < 0.05$). Statistical significance was determined using Dunnett's one-tailed test (ANOVA).

Table 2. Effects of E2 and E2 plus methyl-substituted DIMs on uterine activities in immature female B6C3F1 mice

Compound	Uterine wet weight (mg) (% E2 act)	PR (fmol/uterus) (% E2 act)	UPO (units/mg) (% E2 act)
Corn oil	11.9 ± 1.8	533 ± 153	0.19 ± 0.01
E2 (0.02 μg/mouse)	41.0 ± 5.2	2186 ± 188	2.57 ± 0.01
E2 + 1,1′-diMeDIM	36.9 ± 3.4 (90)*	1724 ± 212 (79)*	1.77 ± 0.02 (69)*
Corn oil	12.1 ± 1.7	350 ± 47	0.19 ± 0.01
E2 (0.02 μg/mouse)	30.1 ± 3.3	1568 ± 100	1.14 ± 0.04
E2 + 2,2′-diMeDIM	29.1 ± 2.3 (97)	1044 ± 38 (67)*	0.98 ± 0.01 (86)*
Corn oil	11.3 ± 2.6	218 ± 36	0.14 ± 0.02
E2 (0.02 μg/mouse)	36.2 ± 7.6	1520 ± 91	1.38 ± 0.01
E2 + 5,5′-diMeDIM	35.2 ± 4.5 (97)	1118 ± 59 (74)*	0.79 ± 0.04 (86)*
Corn oil	13.4 ± 3.5	111 ± 282	0.17 ± 0.00
E2 (0.02 μg/mouse)	31.6 ± 6.1	1356 ± 519	1.07 ± 0.8
E2 + 1,1′,2,2′-tetrameDIM	31.8 ± 6.7 (101)	608 ± 387 (45)*	1.26 ± 0.01 (118)

Mice were treated with corn oil (control), E2 alone or in combination with the methyl-substituted DIMs for three successive days and uterine wet weight, PR binding activity, and peroxidase activity (UPO) were determined as described in the Materials and methods.
*Cotreatments that are significantly different from E2-treated groups ($p < 0.05$). Statistical significance was determined using Dunnett's one-tailed test (ANOVA). Cotreatments = E2 + 100 mg/kg substituted DIM.
This Table summarizes results from four separate experiments.

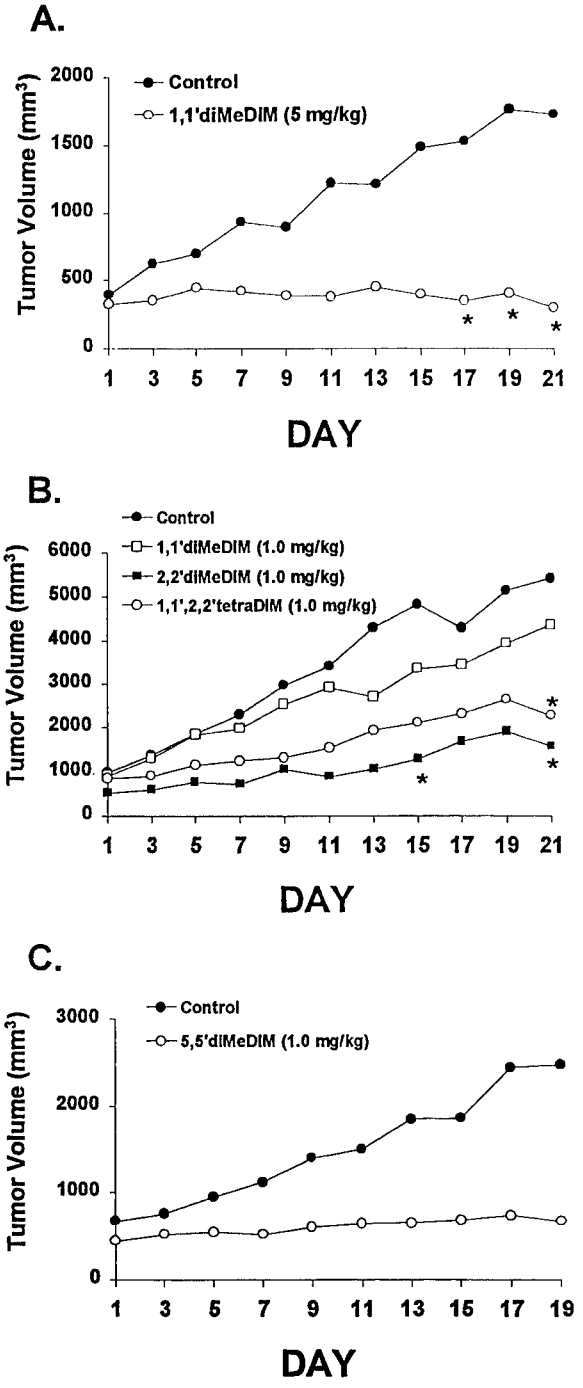


Figure 5. Antitumorigenic activity of methyl-substituted DIMs in rats bearing DMBA-induced mammary tumors. Tumors were allowed to reach a small predetermined size before the initiation of treatments, every other day for 19 (A and B) or 17 (C) days by gavage (in 2 mg/kg corn oil). *Significant inhibition of tumor volume compared to control-treated animals.

for potential clinical use in treatment of breast cancer, namely alternate-substituted alkyl PCDFs and dihaloDIMs [30–32]. Recent studies reported that 5,5′-dibromo-, 4,4′-dichloro- and 6,6′-dichloroDIM inhibited mammary tumor growth in DMBA-induced female Sprague-Dawley rats at a dose of 1 mg/kg every second day (X10) [32]. It has previously been shown that replacement of halogens with methyl or alkyl substituents in alternate-substituted PCDFs and related compounds tends to reduce toxicity [33] without affecting antiestrogenic/antitumorigenic activity [34–37], and therefore this study investigates a series of DIMs containing 2–4 methyl groups and no halogen substituents.

Results with the methyl-substituted DIMs showed that they interact with and transform the cytosolic AhR. However, none of these compounds induced CYP1A1-dependent EROD activity (data not shown) or reporter gene activity in T47D cells transfected with Ah-responsive pRNH11c (Figure 3). Thus, the methyl-substituted DIMs exhibit minimal AhR agonist/antagonist activities with respect to modulation of CYP1A1, and similar results were observed for dihalo-substituted DIMs [32]. The AhR antagonist and agonist activities of DIM were generally observed at higher doses (30–100 μM) [23, 31]; however, the methyl-substituted DIMs were more cytotoxic and therefore were only tested at concentrations $\leq 10\,\mu M$. Interestingly, the results of cell proliferation studies using T47D human breast cancer cells demonstrated that all methyl-substituted DIMs inhibited E2-induced cell proliferation at concentrations from 0.1 to 10 μM and therefore, their effective doses as antiestrogens were lower than concentrations required for induction of CYP1A1-dependent activities.

Previous studies showed that SAhRMs and other AhR agonists inhibit E2-induced responses in the rodent uterus, and some inhibitory effects were also observed for four of the more active (*in vitro*) methyl-substituted DIMs at a dose of 100 mg/kg/day (X3) (Tables 1 and 2). The rodent uterus is generally the least sensitive organ for observing inhibitory AhR-ER crosstalk [32, 34–37], and this was consistent with results obtained for both the compounds investigated in this study and the dihaloDIMs [32]. Interestingly, the high dose antiestrogenic responses observed for DIM and related compounds in the rodent uterus (300 mg/kg – total dose) was not accompanied by induction of hepatic CYP1A1-dependent EROD activity which is usually induced in concert with AhR-mediated toxic responses in the rodent.

Table 3. Effects of methyl-substituted DIMs on (A) body weight and organ weights (expressed as percent body weight) and (B) tumor growth in DMBA-induced female Sprague-Dawley rats

(A)	Body wt (g)	Liver wt	Uterine wt	Heart wt	Spleen wt	Kidney wt
Control	268 ± 2	3.4 ± 0.2	0.19 ± 0.01	0.38 ± 0.01	0.30 ± 0.02	0.34 ± 0.17
1,1′-diMeDIM[a]	264 ± 8	3.0 ± 0.2	0.20 ± 0.02	0.39 ± 0.02	0.25 ± 0.01	0.35 ± 0.02
Control	252 ± 5	3.8 ± 0.3	0.24 ± 0.07	0.41 ± 0.02	0.41 ± 0.06	0.41 ± 0.02
2,2′-diMeDIM[b]	255 ± 8	3.3 ± 0.1	0.16 ± 0.01	0.40 ± 0.02	0.29 ± 0.3	0.38 ± 0.02
1,1′-diMeDIM[b]	258 ± 6	3.7 ± 0.2	0.19 ± 0.02	0.41 ± 0.02	0.36 ± 0.02	0.52 ± 0.01
1,1′,2,2′-teMeDIM[b]	260 ± 9	3.6 ± 0.2	0.17 ± 0.02	0.41 ± 0.02	0.33 ± 0.06	0.37 ± 0.01
Control	271 ± 9	3.7 ± 0.2	0.27 ± 0.05	0.41 ± 0.01	0.22 ± 0.02	0.85 ± 0.02
5,5′-diMeDIM[b]	273 ± 7	4.3 ± 0.2	0.23 ± 0.05	0.41 ± 0.01	0.25 ± 0.01	0.91 ± 0.05

(B)	Final tumor volume (% control)	Tumor weight (% control)	Rate of tumor growth (mm^3/day)	Hepatic EROD activity (pmol/min/mg)
Control	100 ± 24	100 ± 21	69 ± 4	252 ± 26
1,1′-diMeDIM[a]	18 ± 5*	19 ± 5*	0 ± 2	294 ± 80
Control	100 ± 31	100 ± 27	233 ± 15	291 ± 97
1,1′-diMeDIM[b]	75 ± 33	113 ± 73	162 ± 8	384 ± 97
2,2′-diMeDIM[b]	29 ± 9*	39 ± 15*	65 ± 8*	385 ± 81
1,1′,2,2′-teMeDIM[b]	40 ± 22*	55 ± 24	91 ± 7*	347 ± 101
Control	100 ± 49	ND	106 ± 6	ND
5,5′-diMeDIM[b]	27 ± 24	ND	43 ± 5*	ND

Fifty-day-old female Sprague-Dawley rats were initiated with DMBA and, when mammary tumors first appeared, animals were treated with corn oil (control), [a]1,1′-diMeDIM (5 mg/kg), or [b]methyl-substituted DIMs (1 mg/kg) every second day (X10) as described in the Materials and methods.
*Significantly different from control.
ND = not determined.

In the DMBA-induced rat mammary tumor model, three of the four congeners investigated in this study, namely 2,2′-diMeDIM, 5,5′-diMeDIM and 1,1′,2,2′-tetraMeDIM, inhibited mammary tumor growth at a dose of 1 mg/kg, and this was not accompanied by changes in hepatic EROD activity, organ weights/histopathology or body weight. Thus, like DIM and the dihaloDIMs [31, 32], methyl-substituted analogs also exhibit both antiestrogenic and antitumorigenic activity and represent the first group of synthetic SAhRMs that do not contain halogen substitution.

DIM is not directly a phytochemical; however, after consumption of cruciferous vegetables, I3C conjugates are hydrolyzed to give I3C which in turn undergoes self-condensation in the acidic environment of the gut to give several products including DIM [22]. Many of these I3C condensation products are AhR agonists [22, 28], and our studies have focused on using DIM as a model for development of clinically useful SAhRMs. Other I3C condensation products such as 2-(indol-3-ylmethyl)-3,3′- indolylmethane (a trimer- of I3C) also show promise for *in vitro* inhibition of breast cancer cell growth [38], whereas another major trimeric condensation product, 5,6,11,12,17,18-hexahydrocyclononal[1,2-b′:7,8-b″]triindole is estrogenic [39]. In contrast to I3C, DIM is stable in an acidic environment and does not form additional condensation products, and we are developing analytical methods for metabolic and pharmacokinetic studies of DIM and some of the more potent antitumorigenic derivatives identified in this study. These studies are not possible for I3C due to its instability. Current research is also focused on evaluating the long term animal toxicology of DIMs for selection of candidate compounds for clinical applications.

Acknowledgements

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References

1. Sondik EJ: Breast cancer trends. Incidence, mortality, and survival. Cancer 74: 995–999, 1994
2. Hulka BS, Liu ET, Lininger RA: Steroid hormones and risk of breast cancer. Cancer 74: 1111–1124, 1994
3. Hulka BS: Epidemiologic analysis of breast and gynecologic cancers. In: Aldaz M, Gould MN, McLachlan J, Slaga TJ (eds) Etiology of Breast and Gynecological Cancers. Wiley-Liss, 1997, pp 17–29
4. Jordan VC: Molecular mechanisms of antiestrogen action in breast cancer. Breast Cancer Res Treat 31: 41–52, 1994
5. McDonnell DP: The molecular pharmacology of SERMs. TEM 10: 301–311, 1999
6. Smith CL, O'Malley BW: Evolving concepts of selective estrogen receptor action: from basic science to clinical applications. TEM 10: 299–300, 1999
7. Jordan VC: Targeted antiestrogens to prevent breast cancer. TEM 10: 312–317, 1999
8. Moon RC, Mehta RG, Detrisac CJ: Retinoids as chemopreventive agents for breast cancer. Cancer Detect Prev 16: 73–79, 1992
9. Bollag W: Experimental basis of cancer combination chemotheraphy with retinoids, cytokines, 1,25-dihydroxyvitamin D_3, and analogs. J Cell Biochem 56: 427–435, 1994
10. Bischoff ED, Heyman RA, Lamph WW: Effect of the retinoid X receptor-selective ligand LGD1069 on mammary carcinoma after tamoxifen failure. J Natl Cancer Inst 91: 2118–2118, 1999
11. Suh N, Wang Y, Williams CR, Risingsong R, Gilmer T, Willson TM, Sport MB: A new ligand for the peroxisome proliferator-activated receptor-γ (PPAR-γ), GW7845, inhibits rat mammary carcinogenesis. Cancer Res 59: 5671–5673, 1999
12. Safe S: 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and related environmental antiestrogens: characterization and mechanism of action. In: Naz RK (ed) Endocrine Disruptors. CRC Press, Boca Raton, FL, 1999, pp 187–221
13. Safe S, Qin C, McDougal A: Development of selective aryl hydrocarbon receptor modulators (SARMs) for treatment of breast cancer. Expert Opin Invest Drugs 8: 1385–1396, 1999
14. Zacharewski T, Safe S: Antiestrogenic activity of TCDD and related compounds. In: Korach KS (ed) Reproductive and Developmental Toxicology. Marcel Dekker, New York, 1998, pp 431–448
15. Porter W, Safe S: Estrogenic and antiestrogenic compounds. In: Puga A, Wallace KB (eds) Molecular Biology Approaches to Toxicology. Techbooks, Fairfax, VA, 1998, pp 267–283
16. Ema M, Sogawa K, Watanabe N, Chujoh Y, Matsushita N, Gotoh O, Funae Y, Fujii-Kuriyama Y: cDNA cloning and structure of the putative Ah receptor. Biochem Biophys Res Comm 184: 246–253, 1992
17. Burbach KM, Poland AB, Bradfield CA: Cloning of the Ah-receptor cDNA reveals a distinctive ligand-activated transcription factor. Proc Natl Acad Sci USA 89: 8185–8189, 1992
18. Hoffman EC, Reyes H, Chu F-F, Sander F, Conley LH, Brooks BA, Hankinson O: Cloning of a factor required for activity of the Ah (dioxin) receptor. Science 252: 954–958, 1991
19. Wilson CL, Safe S: Mechanisms of ligand-induced aryl hydrocarbon receptor-mediated biochemical and toxic responses. Toxicol Pathol 26: 657–671, 1998
20. Probst MR, Hankinson O: Interactions of ARNT with AhR and HIF-1α. In: Puga A, Wallace KB (eds) Molecular Biology of the Toxic Response. Taylor and Francis, Philadelphia, 1999, pp 377–392
21. Whitlock JP, Okino ST, Dong L, Ko HP, Clarke-Katzenberg R, Ma Q, Li H: Induction of cytochrome P4501A1: a model for analyzing mammalian gene transcription. FASEB J 10: 809–818, 1996
22. Bjeldanes LF, Kim JY, Grose KR, Bartholomew JC, Bradfield CA: Aromatic hydrocarbon responsiveness-receptor agonists generated from indole-3-carbinol *in vitro* and *in vivo* – comparisons with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Proc Natl Acad Sci USA 88: 9543–9547, 1991
23. Chen I, Safe S, Bjeldanes L: Indole-3-carbinol and diindolylmethane as aryl hydrocarbon (Ah) receptor agonists and antagonists in T47D human breast cancer cells. Biochem Pharmacol 51: 1069–1076, 1996
24. Casper RF, Quesne M, Rogers IM, Shirota T, Jolivet A, Milgrom E, Savouret JF: Resveratrol has antagonist activity on the aryl hydrocarbon receptor: implications for prevention of dioxin toxicity. Mol Pharmacol 56: 784–790, 1999
25. Seidel SD, Li V, Winter GM, Rogers WJ, Martinez EI, Denison MS: Ah receptor-based chemical screening bioassays: application and limitations for the detection of Ah receptor agonsists. Toxicol Sci 55: 107–115, 2000
26. Gasiewicz TA, Kende AS, Rucci G, Whitney B, Willey JJ: Analysis of structural requirements for Ah receptor antagonist activity: ellipticines, flavones, and related compounds. Biochem Pharmacol 52: 1787–1803, 1996
27. Gradelet S, Astorg P, Pineau T, Canivenc MC, Siess MH, Leclerc J, Lesca P: Ah receptor-dependent CYP1A induction by two carotenoids, canthaxanthin and β-apo-8′-carotenal, with no affinity for the TCDD binding site. Biochem Pharmacol 54: 307–315, 1997
28. Jellinck PH, Forkert PG, Riddick DS, Okey AB, Michnovicz JJ, Bradlow HL: Ah receptor binding properties of indole carbinols and induction of hepatic estradiol hydroxylation. Biochem Pharmacol 43: 1129–1136, 1993
29. Denison MS, Seidel SD, Rogers WJ, Ziccardi M, Winter GM, Heath-Pagliuso S: Natural and synthetic ligands for the Ah receptor. In: Puga A, Kendall RJ (eds) Molecular Biology Approaches to Toxicology. Taylor and Francis, London, 1998, pp 3–33
30. McDougal A, Wilson C, Safe S: Inhibition of 7,12-dimethylbenz[*a*]anthracene-induced rat mammary tumor growth by aryl hydrocarbon receptor agonists. Cancer Lett 120: 53–63, 1997
31. Chen I, McDougal A, Wang F, Safe S: Aryl hydrocarbon receptor-mediated antiestrogenic and antitumorigenic activity of diindolylmethane. Carcinogenesis 19: 1631–1639, 1998
32. McDougal A, Sethi-Gupta M, Ramamoorthy K, Sun G, Safe S: Inhibition of carcinogen-induced rat mammary tumor growth and other estrogen-dependent responses by symme-

trical dihalo-substituted analogs of diindolylmethane. Cancer Letts 151: 169–179, 2000
33. Romkes M, Piskorska-Pliszczynska J, Keys B, Safe S, Fujita T: Quantitative structure-activity relationships: analysis of interactions of 2,3,7,8-tetrachlorodibenzo-*p*-dioxon and 2- substituted analogues with rat, mouse, guinea pig and hamster cytosolic receptor. Cancer Res 47: 5108–5111, 1987
34. Safe S: Modulation of gene expression and endocrine response pathways by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and related compounds. Pharmacol Therap 67: 247–281, 1995
35. Romkes M, Safe S: Comparative activities of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and progesterone on antiestrogens in the female rat uterus. Toxicol Appl Pharmacol 92: 368–380, 1988
36. Romkes M, Piskorska-Pliszczynska J, Safe S: Effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on hepatic and uterine estrogen receptor levels in rats. Toxicol Appl Pharmacol 87: 306–314, 1987
37. Astroff B, Safe S: Comparative antiestrogenic activities of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and 6-methyl-1,3,8-trichlorodibenzofuran in the female rat. Toxicol Appl Pharmacol 95: 435–443, 1988
38. Chang Y-C, Riby J, Chang GH-F, Peng B-C, Firestone G, Bjeldanes LF: Cytostatic and antiestrogenic effects of 2-(indol-3-ylmethyl)-3,3′-diindolylmethane, a major *in vivo* product of dietary indole-3-carbinol. Biochem Pharmacol 58: 825–834, 1999
39. Riby JE, Feng C, Chang Y-C, Schaldach CM, Firestone GL, Bjeldanes LF: The major tricyclic trimeric product of indole-3-carbinol is a strong agonist of the estrogen receptor signaling pathway. Biochemistry 39: 910–918, 2000

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