AhR-Mediated Antiestrogenicity of Diindolylmethane and Analogs *In vivo* and *In vitro*

Kavita Ramamoorthy, Marisa Navo, Mona S. Gupta and Stephen H. Safe

Department of Veterinary Physiology and Pharmacology, Texas A&M University, College Station, TX 77843-4466

INTRODUCTION

Diindolylmethane (DIM) is the dimerization product of indole-3-carbinol (I3C), an antitumorigenic compound found in cruciferous vegetables. DIM has previously been shown to inhibit 7,12-dimethylbenzanthracene (DMBA)-induced mammary tumors, as well as 17β-estradiol (E_2)-induced cell proliferation in breast cancer cells. Research in this laboratory has shown that the antitumorigenic and antiestrogenic responses are mediated via the aryl hydrocarbon receptor (AhR) (1,2). DIM and several substituted analogs were examined in this study for antiestrogenic activity *in vivo* and *in vitro*. The female B6C3F1 mouse uterine model was used for *in vivo* studies, and effects of E_2 and E_2 + substituted DIMs on progesterone receptor levels, uterine peroxidase activity and uterine wet weight were determined. Rat cytosolic AhR binding and proliferation of T47D breast cancer cells were also investigated. Results indicate that among the substituted analogs, 1,1'-dimethyldiindolylmethane (1-Me-DIM) and 5,5'-dichlorodiindolylmethane (5-Cl DIM) were significantly antiestrogenic in all assays and more potent than DIM.

MATERIALS AND METHODS

Compounds. The following substituted DIMs were synthesized in the laboratory: 1,1'-dimethyldiindolylmethane (1-Me-DIM), 2,2'-dimethyldiindolylmethane (2-Me-DIM), 6,6'-dimethyldiindolylmethane (6-Me-DIM), 5,5'-dichlorodiindolylmethane (5-Cl-DIM), 5,5'-dibromodiindolylmethane (5-Br-DIM), and 6,6'-difluorodiindolylmethane (6-Fl-DIM).

Animals. Twenty-one day old B6C3F1 female mice were purchased from Jackson Laboratories and housed 6-9 per cage with ad libitum access to food and water. DIM and substituted analogs was dissolved in corn oil with slight warming and the total dose divided into 3 daily administrations. Animals were divided into 3 groups of 6-8 animals each and dosed for 3 days on days 21, 22 and 23. One group received vehicle control corn oil (50 μl) by gavage. The second group received 0.02μg/day E_2 (in corn oil) by i.p. injection. The third group received 100 mg/kg DIM or substituted analog by gavage plus 0.02 μg/day E_2 by i.p. injection. The doses of E_2 were the minimal effective dose which significantly induced

the 3 uterine responses of interest. Animals were killed by carbon dioxide asphyxiation 20 h after the last treatment and uteri were quickly removed, cleaned of connective tissue, weighed, nicked, and blotted. The uteri were then bisected into right and left halves, each half containing an entire uterine horn.

***Progesterone Receptor Binding Assay (PR)*.** PR binding was carried out as previously described (3). Analysis was conducted on pooled uteri from each treatment group and levels are reported in fmol per uterus. The assay were carried out in triplicate and results are given as mean ± standard error.

Uterine Peroxidase Assay (UPO). UPO activity was carried out as previously described (3). Analysis was conducted on pooled uteri from each treatment group and enzyme activity was expressed per uterus. The assay was carried out in triplicate and results are given as mean ± standard error.

Cytosolic AhR Binding Assay. Male Sprague-Dawley rats (4-5 weeks old) were sacrificed by CO_2 asphyxiation and cervical dislocation and the livers perfused with ice-cold HEGD (25 mM Hepes, 1.5 mM EDTA, 1 mM dithiothreitol and 10% glycerol (v/v)) buffer. Livers were homogenized in HEGD (3 ml/g tissue) using a Brinkman/Polytron homogenizer. Homogenates were centrifuged at 10,000 g for 10 min (4°C) and the resulting supernatant was centrifuged at 105,000 g for 1 hr (4°C). The resulting pellet was resuspended in 7-9 ml HEGD buffer and protein concentration measured by the method of Bradford (1976). AhR binding was measured using the hydroxylapatite (HAP) assay. HAP was washed twice with HEGD buffer (pH 7.4) and then resuspended in 2 vol of HEGD buffer. Rat hepatic cytosol (3.0 mg/ml) was incubated with 3 nM [^{3}H]TCDD, 3 nM [^{3}H]TCDD plus a 200 fold excess of unlabelled TCDF or [^{3}H]TCDD plus varying concentrations of DIM/substituted analogs for 2 hr @ 20°C in a shaking water bath. The incubation mixture was then added to 100 µl of the HAP suspension in a disposable 13 X 100 mm glass test tube and further incubated for 30 min at 0 - 4°C with gentle shaking every 10 min. HEGD buffer (1.0 ml) containing 0.5% (v/v) Tween 80 was then added and the tubes were vortexed and centrifuged at 1500 rpm for 5 min. The supernatant was decanted and washed (3x) and ethanol (1.0 ml) was added to the HAP pellet . The tube was vortexed and the contents removed with a Pasteur pipette and the bound [^{3}H]TCDD was determined by liquid scintillation counting.

Cell Proliferation Assay. T47D cells were maintained in a minimal essential media (MEM) supplemented with 1.2 g/L bicarbonate, 5% fetal bovine serum (FBS), pH 7.4, 10 ml/L antibiotic solution. Cells were seeded in 6-well plates (50,000/well) in DME-F12 supplemented with 5% FBS treated with dextran-coated charcoal, 1.2 g/L bicarbonate, and 10 ml/L antibiotic solution. After 24 hr, cells were treated with 1 nM E_2 plus varying concentrations of DIM or substituted analogs (0.1 µM - 10 µM) dissolved in DMSO. The medium was changed and cells were redosed every 48 hr. After 14 days, cells were trypsinized, harvested, centrifuged at 200 g for 5 min at 4°C and resuspended in fresh medium. Cells were counted using a Coulter Z1 cell counter (Coulter Electronics, Hialeah, Fl).

RESULTS AND DISCUSSION

1. At a dose of 100 mg/kg DIM, 1-Me-DIM and 5-Cl-DIM each displayed significant antiestrogenic activity in the immature mouse uterine model (Figure 1):
   a) DIM significantly inhibited E_2-induced PR levels and UPO activity, 21% and 10%, respectively. ($p < 0.05$)
   b) 1-Me-DIM significantly inhibited E_2-induced uterine wet weight, PR levels and UPO activity, 10%, 17% and 31%, respectively. ($p < 0.05$)
   c) 5-Cl-DIM significantly inhibited E_2-induced PR levels and UPO activity, 62% and 41%, respectively. ($p < 0.01$) Uterine wet weight was inhibited 12%, however it was not statistically significant.

2. Competitive AhR binding assays were performed with rat liver cytosol. DIM and all substituted DIMs displaced 3 nM [^{3}H]TCDD from the AhR, indicating competitive binding to the receptor. Binding curves for DIM, 1-Me-DIM, 5-Cl-DIM (Figure 2), 2-Me-DIM, 6-Fl-DIM (Figure 3), 5-Br-DIM and 6-Me-DIM (Figure 4) are provided. All data show that the substituted analogs bound with a higher affinity to the AhR than DIM alone. The studies indicate an order of binding affinity as follows: 5-Cl-DIM > 6-Fl-DIM > 6-Me-DIM > 5-Br-DIM = 2-Me-DIM > 1-Me-DIM.

3. T47D cell proliferation was induced 5-fold after treatment with 1 nM E_2. DIM, 1-Me-DIM and 5-Cl-DIM all inhibited E_2-induced cell proliferation in a dose dependent manner (Figure 5). IC_{50} values for DIM and 1-Me-DIM were 1.21×10^{-6} M and 2.4×10^{-6} M, respectively. An IC_{50} value for 5-Cl-DIM could not be calculated because all concentrations tested inhibited proliferation greater than 50%.

4. Both *in vivo* and *in vitro* data suggest that DIM and substituted analogs exhibit antiestrogenic activity. Of the analogs tested *in vivo* and *in vitro*, 5-Cl-DIM was the most potent, followed by 1-Me-DIM and the results are consistent with the antitumorigenic activity of DIM in the DMBA-induced rat mammary tumor model.

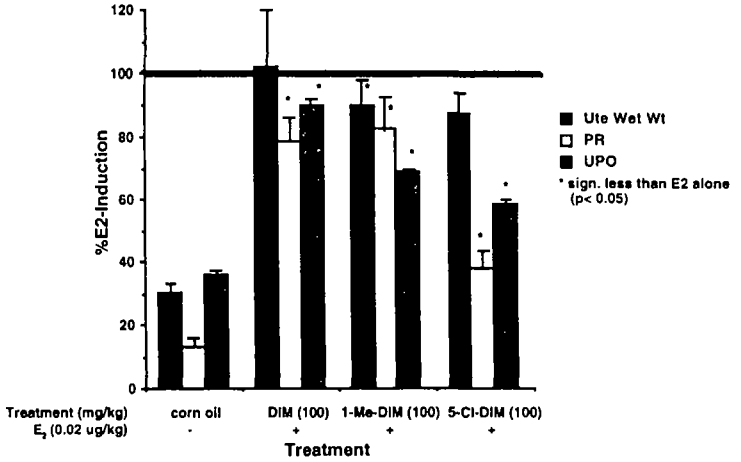

Figure 1. Antiestrogenic effects of DIM, 1-Me-DIM and 5-Cl-DIM on B6C3F1 mouse uterus.

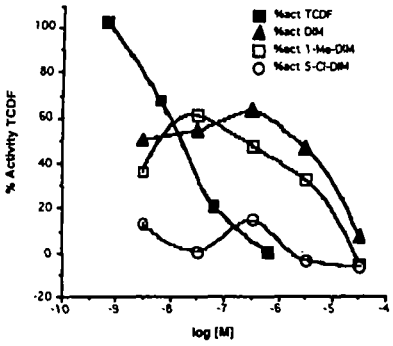

Figure 2. Competitive binding of 1-Me-DIM & 5-Cl-DIM to rat cytosolic AhR.

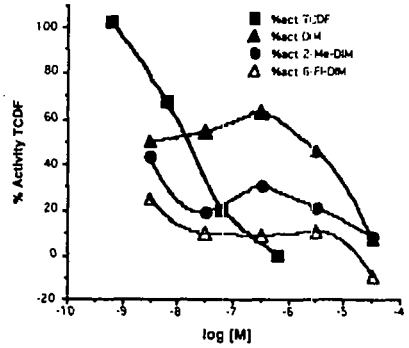

Figure 3. Competitive binding of 2-Me-DIM & 6-Fl-DIM to rat cytosolic AhR.

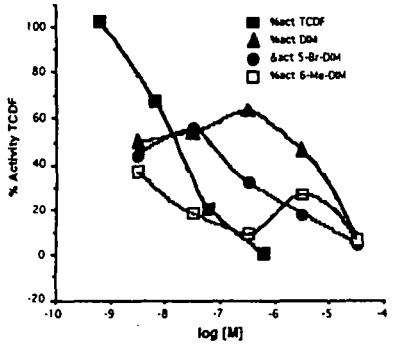

Figure 4. Competitive binding of 5-Br-DIM & 6-Me-DIM to rat cytosolic AhR.

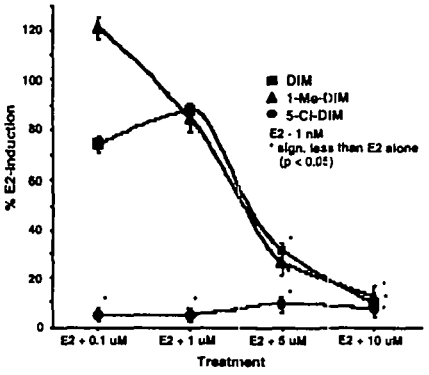

Figure 5. DIM and Substituted DIMs: Inhibition of E2-Induced proliferation of T47D cells.

ACKNOWLEDGEMENTS

The financial assisstance of the State of Texas Advanced Research Program/Advanced Technology Program (ARP/ATP) is gratefully acknowledged.

REFERENCES

1. Chen, I., Safe, S., and Bjeldanes, L. (1996). Indole-3-carbinol and diindolylmethane as aryl hydrocarbon (Ah) receptor agonists and antagonists in T47D human breast cancer cell. ***Bioch. Pharmacol.*** 51(8), 1069-1076.

2. Chen, I., McDougal, A., Wang, F., and Safe, S. (1998). Aryl hydrocarbon receptor-mediated antiestrogenic and antitumorigenic activity of diindolylmethane. ***Carcinogenesis (in review).***

3. Ramamoorthy, K., Wang, F., Chen, I-C., Norris, J., McDonnell, D., Leonard, L., Gaido, K., Bocchinfuso, W., Korach, K., and Safe, S. (1997). Estrogenic activity of a dieldrin/toxaphene mixture in the mouse uterus, MCF-7 human breast cancer cells, and yeast-based estrogen receptor assays: no apparent synergism. ***Endocrinology.*** 138, 1520-1527.