Structure-Ah Receptor Agonist/Binding Activity Relationships of Various Chlorine-Substituted Diindolylmethane Compounds

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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 chlorine-substituted analogs were examined in this study for antiestrogenic and antitumorigenic activity *in vivo*. The female B6C3F1 mouse uterine model was used for *in vivo* antiestrogenicity studies, and effects of E_2 and E_2 + substituted DIMs on progesterone receptor levels, uterine peroxidase activity and uterine wet weight were determined. The 7,12-dimethylbenzanthracene (DMBA) rat mammary tumor model was used to determine antitumorigenic activity of substituted DIMs. Results indicate that 4,4'-Cl_2DIM, 5,5'-Cl_2DIM and 6,6'-Cl_2DIM competitively bound rat AhR, however only 5,5'- and 6,6'-Cl_2DIM were antiestrogenic in the immature mouse uterus. 4,4'-Cl_2DIM and 6,6'-Cl_2DIM but not 5,5'-Cl_2DIM exhibited antitumorigenic activity in the female rat.

Materials and Methods

Compounds. The following substituted DIMs were synthesized in the laboratory: 4,4'-dichlorodiindolylmethane (4,4'-Cl_2DIM), 5,5'-dichlorodiindolylmethane (5,5'-Cl_2DIM), and 6,6'-dichlorodiindolylmethane (6,6'-Cl_2DIM).

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 0.02 μg/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. Assays 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 livers were 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 at 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.

Tumor Studies. Fifty day-old female virgin Sprague-Dawley rats were obtained from Harlan (Houston) and allowed to acclimate for 5 days. On day 54, each rat received 20 mg 7,12-dimethylbenzanthracene dissolved in 0.5 ml corn oil. After the formation of tumors (750 - 1250 mm^3), rats were dosed every other day by oral gavage for 20 days (10 doses) with either corn oil (vehicle) or substituted DIM (1 or 5 mg/kg). Tumor sizes were measured every other day and rats were euthanized on day 21.

Results and Discussion

1. At a dose of 100 mg/kg, both 5,5'-Cl_2DIM and 6,6'-Cl_2DIM significantly inhibited several estrogen-induced markers in the immature mouse uterus (Figure 1):
   a) 5,5'-Cl_2DIM significantly inhibited PR and UPO activity, 62% and 41%, respectively.
   b) 6,6'-Cl_2DIM significantly inhibited uterine wet weight, PR and UPO activity, 16%, 25% and 67%, respectively.
   c) 4,4'-C_2lDIM did not significantly inhibit any of the uterine markers.
   d) No compound significantly induced hepatic cytochrome P450 1A1 activity.

2. Competitive AhR binding assays were performed with rat liver cytosol. All substituted DIMs displaced 3 nM [^{3}H]TCDD from the AhR, indicating competitive binding to the receptor. Binding curves for the compounds are shown in Figure 1. EC50 values are:

4,4'-Cl_2DIM = 6.89 x 10^{-10} M
5,5'-Cl_2DIM = 8.67 x 10^{-9} M
6,6'-Cl_2DIM = 1.99 x 10^{-8} M

indicating a rank order potency as follows: 4,4'-Cl_2DIM > 5,5'-Cl_2DIM > 6,6'-Cl_2DIM. None of the compounds competitively bound the estrogen receptor (data not shown).

3. Studies in the DMBA-induced mammary tumor model indicate that 4,4'-Cl_2DIM and 6,6'-Cl_2DIM were significantly antitumorigenic at a dose of 1.0 mg/kg/2d. 5,5'-Cl_2DIM was not antitumorigenic at 1.0 or 5.0 mg/kg/2d. None of the compounds induced hepatic cytochrome P450 1A1 activity.

4. Data presented herein suggest that chlorine-substituted DIM analogs exhibit antiestrogenic and antitumorigenic activity. Of the analogs tested, 6,6'-Cl_2DIM was the most potent antiestrogen/antitumorigen, inhibiting all three estrogen-induced markers in the immature mouse uterine model and significantly inhibiting tumor growth at a dose as low as 1.0 mg/kg/2d.

Acknowledgements

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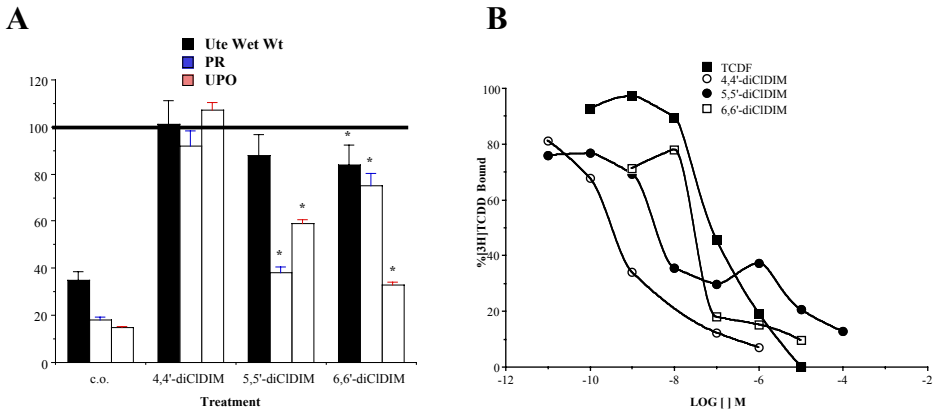

Figure 1. (A) Antiestrogenic activity of chlorine-substituted DIM analogs in the immature mouse uterine model. *Significantly antiestrogenic ($p < 0.05$). (B) Competitive AhR binding activity of chlorine-substituted DIM analogs.

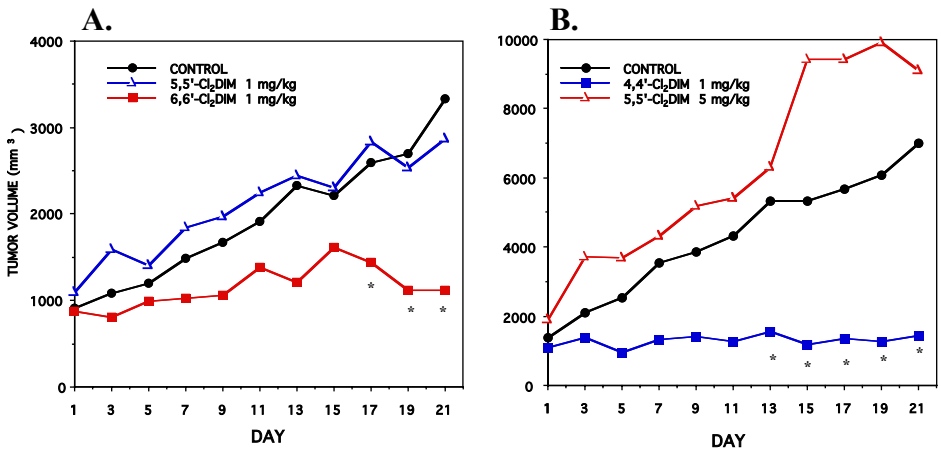

Figure 2. (A) Antitumorigenic activity of 5,5'-Cl_2DIM and 6,6'-Cl_2DIM at 1.0 mg/kg/day in the DMBA-induced rat mammary tumor model. (B) 4,4'-Cl_2DIM at 1.0 mg/kg/day and 5,5'-Cl_2DIM at 5.0 mg/kg/day in the DMBA-induced rat mammary tumor model.

References

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