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Aryl hydrocarbon receptor-mediated antiestrogenic and antitumorigenic activity of diindolylmethane

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Phytochemicals such as indole-3-carbinol (I3C) and sulforaphane are components of cruciferous vegetables which exhibit antitumorigenic activity associated with altered carcinogen metabolism and detoxification. Diindolylmethane (DIM) is a major acid-catalyzed metabolite of I3C formed in the gut that binds to the aryl hydrocarbon receptor (AhR) and treatment of MCF-7 human breast cancer cells with 10–50 μM DIM resulted in rapid formation of the nuclear AhR complex and induction of CYP1A1 gene expression was observed at concentrations >50 μM. Previous studies have demonstrated that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a high affinity AhR ligand, inhibits 17β-estradiol (E2)-induced responses in MCF-7 cells and growth of E2-dependent 7,12-dimethylbenzanthracene (DMBA)-induced mammary tumors in female Sprague–Dawley rats. Results of this study show that like TCDD, DIM inhibits E2-induced proliferation of MCF-7 cells, reporter gene activity in cells transiently transfected with an E2-responsive plasmid (containing a frog vitellogenin A2 gene promoter insert) and down-regulates the nuclear estrogen receptor. Moreover, DIM (5 mg/kg every other day) also inhibits DMBA-induced mammary tumor growth in Sprague–Dawley rats and this was not accompanied by induction of hepatic CYP1A1-dependent activity. Thus, DIM represents a new class of relatively non-toxic AhR-based antiestrogens that inhibit E2-dependent tumor growth in rodents and current studies are focused on development of analogs for clinical treatment of breast cancer.

Introduction

Diet plays an important role in the incidence of various cancers and results of several studies suggest that high fiber, low fat diets combined with high consumption of fruits and vegetables are protective against development of many cancers (1–5). Diets enriched in cruciferous vegetables have been associated with protection against multiple cancers, including breast cancer in women (1–8), and there has been extensive research on the chemoprotective mechanisms associated with phytochemicals produced by these plants. For example, broccoli, broccoli sprouts and cauliflower contain high levels of isothiocyanate conjugates of compounds, such as 4-methylsulfinylbutyl isothiocyanate (sulforaphane), which are potent inducers of phase II drug-metabolizing enzymes, such as glutathione *S*-transferase and NAD(P)H:quinone reductase (9–11). Both of these phase II enzymes play an important role in detoxification of chemical toxins and carcinogens and, not surprisingly, administration of extracts enriched in these enzyme inducers protects against 7,12-dimethylbenzanthracene (DMBA)-induced mammary carcinogenesis in female Sprague–Dawley rats (11,12). These protective effects involve altered metabolism of DMBA and are observed when isothiocyanate-containing extracts are administered either before or during carcinogen treatment.

Indole-3-carbinol (I3C) is another important phytochemical found in cruciferous vegetables (13) and results of several studies show that I3C also exhibits anticarcinogenic activity (14–23). For example, I3C, related compounds and Brussels sprouts inhibit carcinogen-induced mammary tumors in female Sprague–Dawley rats (20,21); dietary I3C decreases spontaneous mammary tumor incidence in C3H/OuJ mice and also inhibits spontaneous endometrial cancer formation in female Donryu rats (23). I3C, like sulphoraphane, induces phase II drug-metabolizing enzymes, such as epoxide hydrolase, glutathione *S*-transferase, glucuronyl transferase and NAD(P)H:quinone oxidoreductase, and these elevated responses undoubtedly play an important role in metabolic detoxification of toxins and carcinogens (21,24–29). In contrast with sulphoraphane, I3C also induces several phase I cytochrome P450-dependent activities and their corresponding genes, including CYP1A1, CYP1A2, CYP2B1 and CYP3A1 (21,25–34). Phase I drug-metabolizing enzyme activities can protect against some carcinogens/toxins by increasing their rate of oxidative metabolism into less toxic metabolites; however, induction of isozymes such as CYP1A1 or CYP1A2 can enhance toxicity or carcinogenicity of some compounds by increasing their rate of oxidative metabolism into more toxic/carcinogenic metabolites.

I3C is unstable and rapidly undergoes acid-catalyzed oligomerization at low pH (and in the gut) to give a complex mixture of products, including diindolylmethane (DIM) (dimer), 5,6,11,12,17,18-hexahydrocyclononal[1,2-b:4,5-b′:7,8-b′′]triindole, [2-(indol-3-ylmethyl)-indol-3-yl]indol-3-yl-methane, 3,3′-bis(indol-3-ylmethyl)indolenine, cyclic and linear tetramers of I3C and indolo[3,2-b]carbazole (ICZ) (25,29,34,35). I3C binds weakly to the aryl hydrocarbon receptor (AhR) and the higher molecular weight condensation products exhibit increased binding affinity for this receptor (33,35). Research in this laboratory has focused on characterization and mechanisms of action of AhR-mediated antiestrogenic activity in the rodent uterus and mammary and human breast cancer cell

Abbreviations: Ah, aryl hydrocarbon; AhR, aryl hydrocarbon receptor; BrdU, bromodeoxyuridine; CAT, chloramphenicol acetyltransferase; DIM, diindolylmethane; DMBA, 7,12-dimethylbenzanthracene; DME F-12, Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham; DMSO, dimethylsulfoxide; DRE, dioxin-responsive element; E2, 17β-estradiol; ER, estrogen receptor; ERE, estrogen response element; EROD, ethoxyresorufin *O*-deethylase; FCS, fetal calf serum; hER, human ER; I3C, indole-3-carbinol; ICZ, indolo[3,2-*b*]carbazole; MCDF, 6-methyl-1,3,8-trichlorodibenzofuran; MEM, minimum essential medium; PBS, phosphate-buffered saline; PCDFs, polychlorinated dibenzofurans; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

lines (reviewed in ref. 36). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), a widely used prototypical AhR ligand, exhibits potent antiestrogenic activity in both *in vivo* and *in vitro* models; moreover, many of these same responses have also been observed for other structural classes of AhR agonists, including polynuclear aromatic hydrocarbons, alternate-substituted alkyl polychlorinated dibenzofurans (PCDFs) and ICZ (36–39).

DIM is an acid-catalyzed metabolite (dimer) of I3C and dietary administration of DIM (5 mmol) 20 h prior to treatment with DMBA inhibits tumor formation in the carcinogen-induced rat mammary tumor model (21); however, these anticarcinogenic effects are related to altered carcinogen metabolism (20–23). Previous studies show that DIM binds the AhR (33,35) and is a weak AhR agonist for induction of *CYP1A1* gene expression in T47D cells (40). This study demonstrates that DIM is an AhR agonist and induces *CYP1A1* gene expression in MCF-7 cells at concentrations from 50 to 100 μM; in contrast, antiestrogenic responses are observed at concentrations as low as 0.1 μM. Moreover, at doses as low as 5 mg/kg every second day, DIM is a potent inhibitor of DMBA-induced mammary tumor growth in female Sprague–Dawley rats. Thus, DIM represents a new class of relatively non-toxic AhR-based antiestrogens with potential for clinical treatment of breast cancer.

Materials and methods

Chemicals and biochemicals

DMBA was purchased from Sigma (St Louis, MO); DIM, [^{3}H]TCDD and TCDD were synthesized in this laboratory to >98% purity. DIM was carefully stored in the dark to avoid photodecomposition. Estrogen receptor (ER) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and AhR antibodies were kindly provided by Drs Carol Holtzapple and Larry Stanker (USDA Agricultural Research Service, College Station, TX). The Vit–CAT construct contains the –821 to –87 promoter insert from the frog vitellogenin A2 gene and was kindly provided by Drs Klein-Hitpass and Ryffel (Institute for Cell Biology, Universitatsklinikum, Essen, Germany) (41). The human ER (hER) expression plasmid was kindly provided by Dr M.Jer Tsai (Baylor College of Medicine, Houston, TX). DMBA, Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham (DME F-12) without phenol red, phosphate-buffered saline (PBS), acetyl-CoA, E2 and 100× antibiotic/antimycotic solution were purchased from Sigma. Fetal calf serum (FCS) was obtained from Intergen (Purchase, NY). Minimum essential medium (MEM) was purchased from Life Technologies (Grand Island, NY). [γ-^{32}P]ATP (3000 Ci/mmol) and [^{14}C]chloramphenicol (53 mCi/mmol) were purchased from NEN Research Products (Boston, MA). Poly(dIdC) and T4 polynucleotide kinase were purchased from Boehringer Mannheim (Indianapolis, IN). The estrogen response element (ERE), mutant ERE, dioxin-responsive element (DRE) and mutant DRE were synthesized by the Gene Technologies Laboratory at Texas A&M University. All other chemicals and biochemicals were the highest quality available from commercial sources.

Cell culture

MCF-7 cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD) and benzo[*a*]pyrene-resistant cells (MCF^{Bapr}) were isolated in this laboratory. Cells were grown in MEM supplemented with 10% FCS plus $NaHCO_3$ (2.2 g/l), gentamycin (2.5 mg/l), penicillin/streptomycin (10 000 units/l and 10 mg/l), amphotericin B (1.25 mg/l) and insulin (10 μg). Cells were maintained in 150 cm^2 tissue culture flasks/plates and incubated at 37°C in a humidified mixture of 5% CO_2 and 95% air under atmospheric pressure.

Cell proliferation assay

Cells were seeded at 7.5×10^4 cells/well in 6-well plates in medium containing 2 ml DME F-12 without phenol red, supplemented with 5% FCS treated with dextran-coated charcoal as previously described (38). After 24 h, the medium was changed and cells were treated with 17β-estradiol (E2), DIM or E2 plus DIM for 11 days. The medium was changed and cells were redosed every 48 h. The cells were harvested and counted using a Coulter Z1 cell counter. All determinations were carried out in triplicate and results are expressed as means ± SD.

Transient transfection and chloramphenicol acetyltransferase (CAT) assays

The plasmid containing the vitellogenin A2 gene promoter was fused to the bacterial *cat* reporter gene (41). Cells were seeded in 100 mm dishes and grown until 70% confluent; 5 μg plasmid and 2 M $CaCl_2$ were used for the assay. After 16 h, cells were treated with 1 nM E2, 1 nM TCDD and 10–50 μM DIM for 48 h. Cells were then washed with PBS and scraped from the plates. Cell lysates were prepared in 0.16 ml 0.25 M Tris–HCl, pH 7.5, by three freeze–thaw sonication cycles (3 min each). Cell lysates were incubated at 56°C for 7 min to remove endogenous deacetylase activity. CAT activity was determined using 0.2 mCi *d*-threo-[dichloroacetyl-1-^{14}C]chloramphenicol and 4 mM acetyl-CoA as substrates. The protein concentrations were determined using bovine serum albumin as the standard. Following thin layer chromatography, acetylated products were visualized and quantitated using a Betascope 603 Blot analyzer. CAT activity was calculated as the percentage of activity in cells treated with dimethylsulfoxide (DMSO) alone and results are expressed as means ± SD. Experiments were carried out at least in triplicate.

Preparation of cytosolic and nuclear extracts

Cells were incubated for 1.5 or 12 h with DMSO, 10 nM TCDD or 10–50 μM DIM. Cells were harvested, then washed twice in 20 ml HEGD buffer (25 mM HEPES, 1.5 mM EDTA, 10% glycerol, 1.0 mM dithiothreitol, pH 7.6). The washed cell pellet was resuspended in 3 ml HED (as HEGD buffer without the glycerol) and incubated for 10 min. After incubation, cells were pelleted and resuspended with an additional 1.5 ml HEDG buffer and homogenized using a tight Teflon pestle/drill apparatus. Homogenates were centrifuged at 1500 *g* for 10 min. Supernatants were collected and then centrifuged at 105 000 *g* for 30 min at 4°C. The supernatants representing cytosolic extracts were then stored at –80°C until used. The pelleted fraction was resuspended in 3 ml HEGD buffer containing 0.5 M KCl, pH 8.5, and allowed to stand at 4°C for 1 h and then centrifuged at 105 000 *g* for 30 min at 4°C. The supernatants containing nuclear extracts were stored at –80°C until further used. Nuclei prepared by this method were found to be intact as determined by microscopic examination and trypan blue staining.

Western blot analysis

Cytosolic and nuclear extracts from MCF-7 cells (200 μg) were separated on 10% SDS gels and transferred to PVDF membrane. The membrane was blocked for 1 h with 5% milk in PBS (blocking buffer). Anti-ER (mouse monoclonal IgGa, 0.1 μg/ml) and anti-AhR (Rpt monoclonal antibody, 1 μg/ml) antibodies were added to the blocking buffer and incubated for 1 and 2.5 h respectively with gentle shaking. The blots were washed (three times) for 5 min with 0.05% Tween 20 in PBS (rinse buffer). IgG anti-mouse peroxidase conjugate (1:1000 dilution) was added to the rinse buffer and incubated for 1 h and 20 min for ER and AhR respectively. After washing (three times) for 5 min, bound antibodies were detected with an ECL Western Blotting Kit (Amersham Life Science, Arlington Heights, IL).

Gel electrophoretic mobility shift assay

Ten picomoles of synthetic human DRE or ERE oligonucleotide were labeled at the 5′-end using T4 polynucleotide kinase and [^{32}P]ATP. For the AhR:DRE or ER:ERE binding assays nuclear extracts (10 μg) from control (DMSO), 10 nM TCDD- or 50 μM DIM-treated cells were incubated in HEGD buffer with 1 μg poly(dIdC) for 10 min at 20°C to bind non-specific DNA-binding proteins. A 100-fold excess of unlabeled wild-type and mutant DRE or ERE was added for the competition experiments and incubated at 20°C for 5 min. Following addition of ^{32}P-labeled DNA, the mixture was incubated for an additional 15 min at 20°C. Reaction mixtures were loaded onto a 5% polyacrylamide gel and electrophoresed at 110 V in 0.9 M Tris–borate and 2 mM EDTA, pH 8.0. Gels were dried and protein–DNA complexes were visualized by autoradiography.

Northern blot analysis

Cells were plated into 100 mm Petri dishes and, when 60% confluent, treated with DMSO and the test chemicals (DIM or TCDD) for 12 h. A solution of RNAzol™ B (TEL-TEST, Friendswood, TX) was added and cells were scraped from the plates and RNA was isolated as previously described (38). The murine *CYP1A1* cDNA probe was obtained from ATCC. The plasmid pGMB1.1 was a gift from Dr Don Cleveland (Johns Hopkins University) and carries the mouse β-tubulin cDNA cloned into the *Eco*RI site of pGMB1.1. Cellular RNA was separated by electrophoresis and hybridized with [^{32}P]cDNA. CYP1A1 mRNA levels were standardized relative to β-tubulin mRNA in the same sample and band intensities were quantitated on a Betagen Betascope 603 blot analyzer or by autoradiography as previously described (38,40).

DMBA-induced rat mammary carcinoma model

Mammary tumors were induced in virgin 50-day-old Sprague–Dawley rats with a single dose by gavage of 20 mg DMBA (39). After 30–60 days, carcinomas could be detected by palpation in the ductal tubes of the mammary

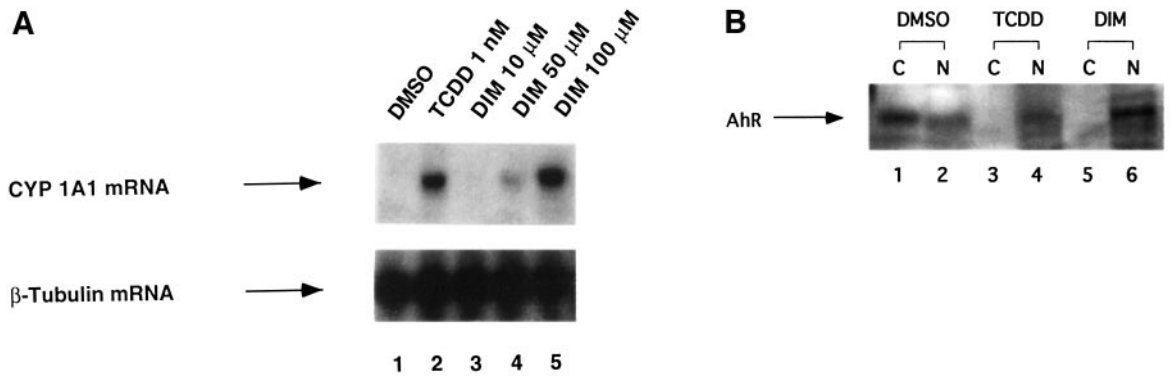

Fig. 1. Effects of DIM and TCDD on CYP1A1 expression and AhR localization in MCF-7 cells. (**A**) Induction of CYP1A1 mRNA levels. MCF-7 cells were treated with TCDD (1 nM) or DIM (10, 50 or 100 μM) for 12 h and CYP1A1 mRNA levels were determined as described in Materials and methods. CYP1A1 mRNA levels were standardized relative to β-tubulin mRNA and the values compared with DMSO treatment (arbitrarily set at 100) (means ± SD for three determinations) were: 100 ± 12, 1309 ± 212, 185 ± 109, 538 ± 335 and 1320 ± 379 respectively (lanes 1–5 respectively). Quantitation of induced bands was determined using a Betagen Betascope 603 blot analyzer and significant induction was observed for 1 nM TCDD and 50 and 100 μM DIM. (**B**) Immunoreactive nuclear AhR protein. MCF-7 cells were treated with DMSO, 10 nM TCDD or 50 μM DIM for 1.5 h and immunoreactive cytosolic and nuclear AhR complex was determined as described in Materials and methods. Treatment with TCDD or DIM resulted in almost complete translocation of the AhR into the nuclear fraction.

glands. After the largest tumor reached a small predetermined volume of 100–200 mm^3, rats were dosed every other day by oral gavage for 20 days (10 doses) with either corn oil (vehicle) or DIM (5 mg/kg). The rats were killed on day 21. Tumor sizes were measured biweekly with calipers and volumes calculated using the formula (length/2)×(width/2)×(depth/2)×(4/5×π). Livers were perfused, weighed and used for the ethoxyresorufin *O*-deethylase (EROD) assay. Animals were housed initially in groups of two and isolated after formation of tumors, to prevent cannibalism. Multiple organs were excised, weighed and retained for histopathological examination.

Preparaton of microsomes and determination of EROD activity

Perfused livers were excised, weighed, placed in HEDGM buffer (25 mM HEPES, 1.5 mM EDTA, 1 mM dithiothreitol, 20 mM sodium molybdate and 10% glycerol, pH 7.6), homogenized and centrifuged at 10 000 *g* for 20 min. The lipid layer was removed and the resulting supernatant was recentrifuged at 105 000 *g* for 1 h. The resulting microsomal pellet was resuspended in HEDG buffer, protein concentration was determined (42) and microsomes were stored at –80°C until used. Hepatic microsomal EROD activities were determined by fluorimetric procedures (520/590 nm excitation/emission) as previously described (39). A cofactor solution (0.0087% NADPH and NADH, 0.06% $MgSO_4$ and 0.125% bovine serum albumin in 0.01 M HEPES) was added to the microsomes and measurements were made in triplicate on a 46-well plate using a plate reader (Millipore, Watertown, MA).

UV DNA crosslinking

Ten picomoles of the synthetic oligonucleotide, 5′-GAT CTC CGG TCC TTC TCA CGC AAC GCC TGG GC-3′, was annealed to a 10 pmol 7 bp complementary primer, 5′-GCC CAG G-3′. The annealed template was end-filled with the Klenow fragment of DNA polymerase in the presence of 0.1 μM dGTP, dATP, bromodeoxyuridine (BrdU) and 1 μM [^{32}P]dCTP, and designated as the BrdU-substituted DRE oligonucleotide. Nuclear extracts (10 μg) from MCF-7 cells treated with 50 μM DIM or 10 nM TCDD were incubated with the BrdU-substituted DNA for 15 min at 20°C following a 15 min incubation at 20°C with 400 ng [^{32}P]DRE or poly(dIdC) in HEGD buffer for 10 min, followed by a 5 min incubation at 20°C with unlabeled excess competitors or AhR antibody. Incubation mixtures were irradiated using a FOTODYNE UV transilluminator at >205 nm for 3 min at 20°C. Samples were then mixed with 20 μl SDS loading buffer, heated to 95°C for 5 min and subjected to electrophoresis on SDS–8.5% polyacrylamide gels. Molecular weights of UV crosslinked nuclear ligand–AhR complexes were calculated from [^{14}C]methylated standards obtained from Amersham.

Statistical analysis

The statistical differences between different groups were determined using Student's *t*-test or ANOVA (Scheffe's) and the levels of significance are noted ($P < 0.05$). The results are expressed as means ± SD for at least three replicate determinations for each experiment.

Results

The results in Figure 1A illustrate the concentration-dependent induction of CYP1A1 mRNA levels by 10–100 μM DIM in MCF-7 cells. No significant induction was observed for 10 μM DIM (lane 3); however, at higher concentrations (50 and 100 μM), CYP1A1 mRNA levels were significantly increased (lanes 4 and 5 respectively) and 100 μM DIM induced a response greater than or equal to that observed for 1 nM TCDD (lane 2). The effects of both 10 nM TCDD and 50 μM DIM on the subcellular distribution of immunoreactive AhR in MCF-7 cells were also investigated (Figure 1B). In cells treated with the carrier solvent DMSO, the cytosolic fraction contained relatively high levels of the AhR protein compared with the nuclear fraction (lanes 1 and 2). In contrast, after treatment with 10 nM TCDD or 50 μM DIM, immunoreactive AhR protein was almost exclusively localized in the nuclear fraction (lanes 4 and 6). Ligand-induced formation of the nuclear AhR complex was also confirmed by gel mobility shift assays (Figure 2A); incubation of [^{32}P]DRE with nuclear extracts from cells treated with 10 nM TCDD (lane 2) or 10, 20 or 50 μM DIM (lanes 3–5) gave retarded bands which were competitively decreased by co-incubation with excess unlabeled DRE (lane 6) but not mutant DRE (lane 7). Incubation of [^{32}P]DRE with nuclear extracts from MCF-7 cells treated with DMSO did not form a weak retarded band (lane 1). Both TCDD and DIM induced formation of a nuclear AhR complex in MCF-7 cells which photocrosslinked with BrdU-substituted [^{32}P]DRE to give a 220 kDa complex (Figure 2B, lanes 2 and 6 respectively). Formation of this complex was decreased after co-incubation with 100-fold excess unlabeled DRE (lanes 3 and 7 respectively) or AhR antibody (lanes 4 and 8 respectively), whereas no effects were observed after co-incubation with a non-specific IgG (lanes 5 and 9 respectively). These data demonstrate that both DIM and TCDD are AhR agonists that induce *CYP1A1* gene expression in MCF-7 cells via comparable pathways. Moreover, treatment of MCF-7 cells with 10 μM DIM resulted in formation of a nuclear AhR complex (Figure 2A, lane 3), whereas this was not accompanied by induction of *CYP1A1* gene expression (Figure 1A, lane 3).

The antiestrogenic activity of DIM in MCF-7 cells was also investigated. At concentrations from 0.01 to 10 μM, DIM did not affect proliferation of MCF-7 cells, whereas 1 nM E2 caused a 25-fold increase in cell proliferation (Figure 3A). In cells co-treated with 1 nM E2 plus 0.01–10 μM DIM, there was a concentration-dependent decrease in E2-induced cell

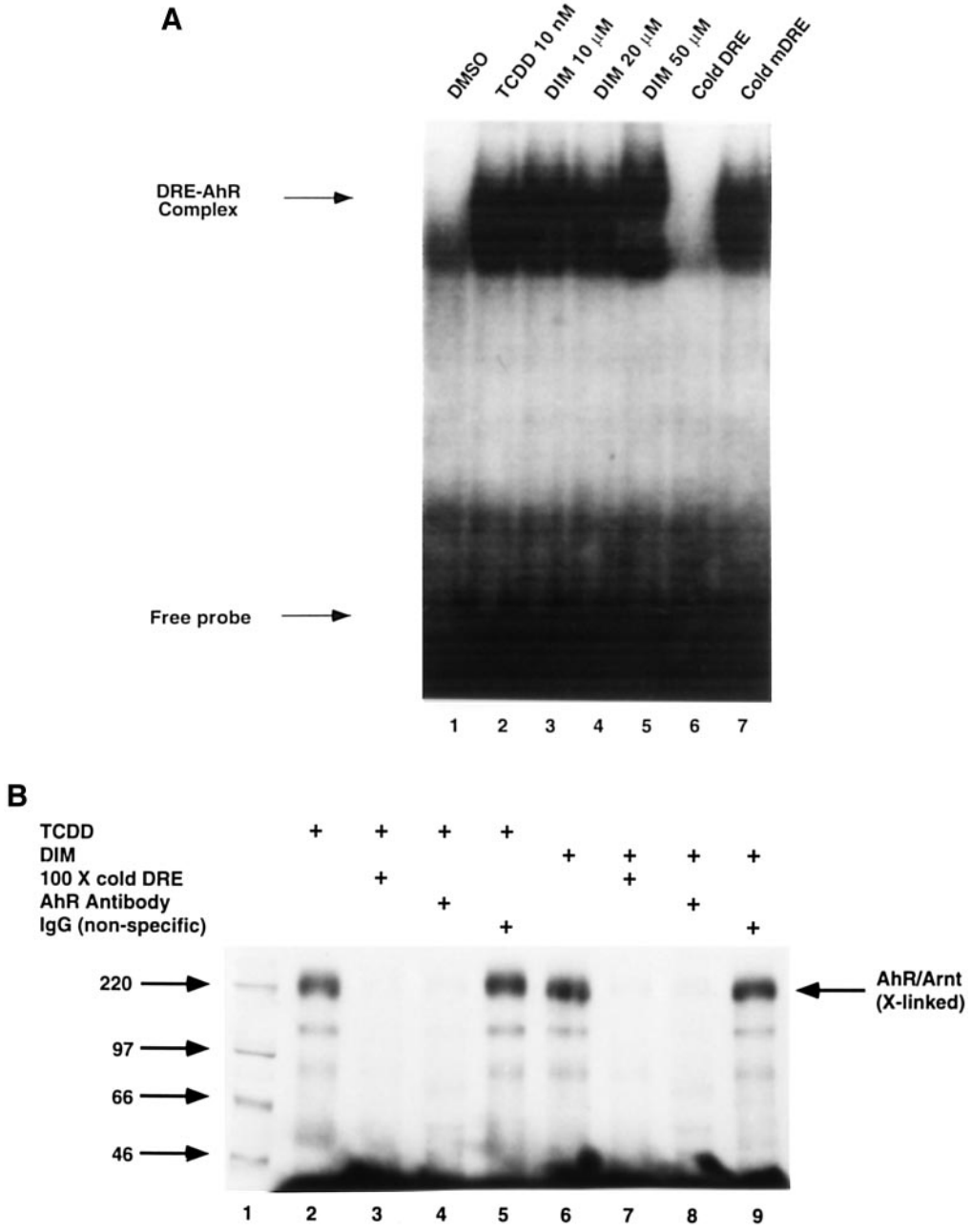

Fig. 2. Ligand-induced formation of the nuclear AhR complex and interaction with DREs. (**A**) Gel retardation analysis of nuclear extracts. MCF-7 cells were treated with DMSO, 10 nM TCDD or 10, 20 or 50 μM DIM for 1.5 h; nuclear extracts were incubated with ^{32}P-labeled DRE, separated by 6% PAGE and visualized by autoradiography as described in Materials and methods. TCDD- and DIM-induced binding of nuclear AhR to [^{32}P]DRE oligonucleotides. Relative intensities of the specifically bound [^{32}P]DRE–AhR complex caused by treatments with DMSO (arbitrarily set at 100), 10 nM TCDD or 10, 20 or 50 μM DIM (lanes 1–5 respectively) were 100 ± 23, 320 ± 30*, 300 ± 24*, 302 ± 23* and 260 ± 51* respectively. The DRE–AhR complex band from TCDD-treated MCF-7 cells was reduced in intensity after incubation with a 25-fold excess of unlabeled DRE (lane 4) but not decreased after co-incubation with excess unlabeled mutant DRE (lane 5). Results are means ± SE of three separate experiments (*significantly increased, $P < 0.05$). (**B**) Crosslinking of nuclear extracts to BrdU-substituted [^{32}P]DRE. Nuclear extracts from MCF-7 cells treated with 10 nM TCDD or 50 μM DIM (as above) were crosslinked with BrdU-substituted [^{32}P]DRE and separated by SDS–PAGE as described in Materials and methods. Both DIM and TCDD induced formation of a specifically bound 220 kDa nuclear AhR complex (lanes 6 and 2 respectively).

proliferation and significant growth inhibition was observed at concentrations from 0.1 to 10 μM. The results summarized in Figure 3B show that in aryl hydrocarbon (Ah)-non-responsive benzo[*a*]pyrene-resistant MCF-7 cells (MCF-7^{BaPr}), DIM alone slightly increased cell growth at the highest concentration (5 μM). In cells co-treated with E2 plus 0.01–5 μM DIM, only minimum inhibition of cell proliferation was observed at 5 μM DIM. The results in Figure 4 illustrate the antiestrogenic activity of TCDD and DIM in MCF-7 cells transiently transfected with the E2-responsive Vit–CAT plasmid. One nanomolar E2 caused a 2.3-fold induction of CAT activity (lane 2), whereas TCDD (lane 3) or DIM alone (lanes 5, 7 and 9) did not significantly induce CAT activity but decreased the response below control (lane 1) values. In cells co-treated with E2 plus 1 nM TCDD (lane 4) or 10, 20 or 50 μM DIM (lanes 5, 7 and 9), there was a significant decrease in hormone-induced CAT activity.

The comparative effects of DIM and TCDD on the nuclear

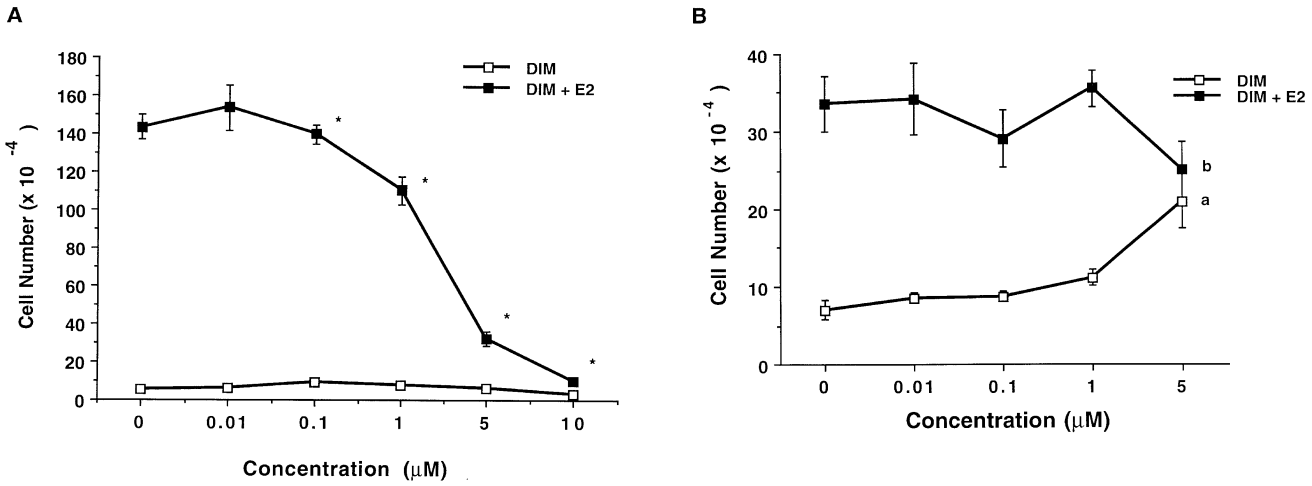

Fig. 3. Inhibition of E2-induced proliferation of wild-type MCF-7 and variant MCF-7^{BaPr} cells by DIM. (**A**) Inhibition of E2-induced proliferation of MCF-7 cells by DIM. MCF-7 cells were treated with 1 nM E2, different concentrations of DIM (□) alone or co-treated with 1 nM E2 plus different concentrations of DIM (■) for 11 days. Cells were harvested and cell number was determined as described in Materials and methods. The results are presented as means of three separate experiments in which SD values were <15%. DIM significantly inhibited (*$P < 0.05$) E2-induced growth of MCF-7 cells at concentrations as low as 0.1 μM. (**B**) Effects of DIM on proliferation of variant MCF-7^{BaPr} cells. MCF-7^{BaPr} cells were treated with 1 nM E2, different concentrations of DIM (□) alone or co-treated with 1 nM E2 plus different concentrations of DIM (■) for 11 days. Cells were harvested and cell number was determined as described in Materials and methods. The results are presented as means of three separate experiments in which SD values were <20%. DIM also induced cell proliferation (a, $P < 0.05$) and significantly inhibited (b, $P < 0.05$) E2-induced growth of MCF-7^{BaPr} cells at a concentration of 5 μM.

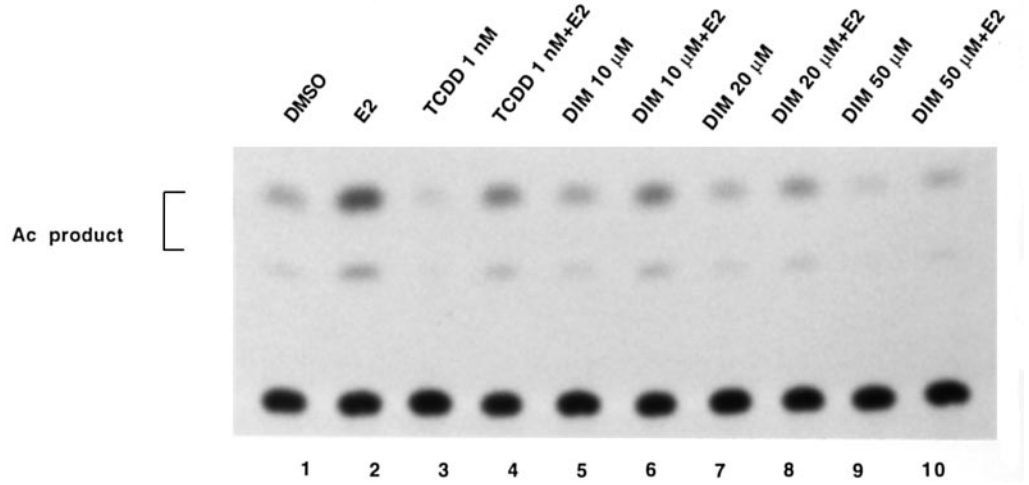

Fig. 4. Inhibition of E2-induced CAT activity in MCF-7 cells by TCDD and DIM. MCF-7 cells were transiently transfected with vitellogenin A2 gene promoter–CAT (2.5 μg) and ER expression plasmids (0.2 μg) and treated with various chemical combinations for 48 h; CAT activity was determined as described in Materials and methods. CAT activity was induced 2.3-fold by 1 nM E2 (lane 2) compared with activity in cells treated with DMSO (control). Relative intensities of acetylated products in cells treated with DMSO (arbitrarily set at 100), 1 nM E2, 1 nM TCDD, 1 nM TCDD plus E2, 10 μM DIM, 10 μM DIM plus E2, 20 μM DIM, 20 μM DIM plus E2, 50 μM DIM and 50 μM DIM plus E2 (lanes 1–10 respectively) were 100 ± 16, 231 ± 11, 33 ± 2, 118 ± 20, 58 ± 10, 127 ± 18, 43 ± 12, 75 ± 28, 40 ± 16 and 71 ± 33% respectively. TCDD (1 nM) and DIM (10–50 μM) significantly inhibited the E2-induced response ($P < 0.05$). Relative intensities (means ± SD for three determinations) of the acetylated products were determined using a Betagen Betascope 603 blot analyzer.

ER were also investigated in MCF-7 cells (Figure 5A). In untreated cells, immunoreactive ER protein was primarily in the nuclear fraction (lanes 1 and 2); however, 1.5 h after treatment with 10 nM TCDD or 50 μM DIM, there was a >70–80% decrease in immunoreactive ER protein (lanes 4 and 6 respectively). In parallel experiments, the binding of [^{32}P]ERE with nuclear extracts from MCF-7 cells treated with 10 nM TCDD (Figure 5B, lane 2) or 10, 20 or 50 μM DIM (lanes 3–5) for 12 h gave less intense retarded bands associated with formation of the ER–ERE complex. ER–ERE retarded band intensity (compared with lane 1) was decreased after co-incubation with unlabeled ERE (lane 6) but not after co-incubation with mutant DRE (lane 7). These data show that DIM, like TCDD, down-regulates the nuclear ER and this was consistent with results of previous studies with TCDD and related AhR agonists (36–38,43).

The antitumorigenic activity of DIM was investigated in female Sprague–Dawley rats initiated with DMBA (Figure 6 and Table I). After initial detection of mammary tumors (100–200 mm^3), animals were treated with either corn oil (control) or DIM (5.0, 1.0 or 0.5 mg/kg) every other day for 20 days. The results showed that 5.0 mg/kg DIM significantly inhibited mammary tumor growth and, at this dose, tumor volumes were similar to those observed on day 1. In contrast, tumor volumes in the control group of rats increased >4-fold over the treatment period, with tumor volume doubling times of 5–7

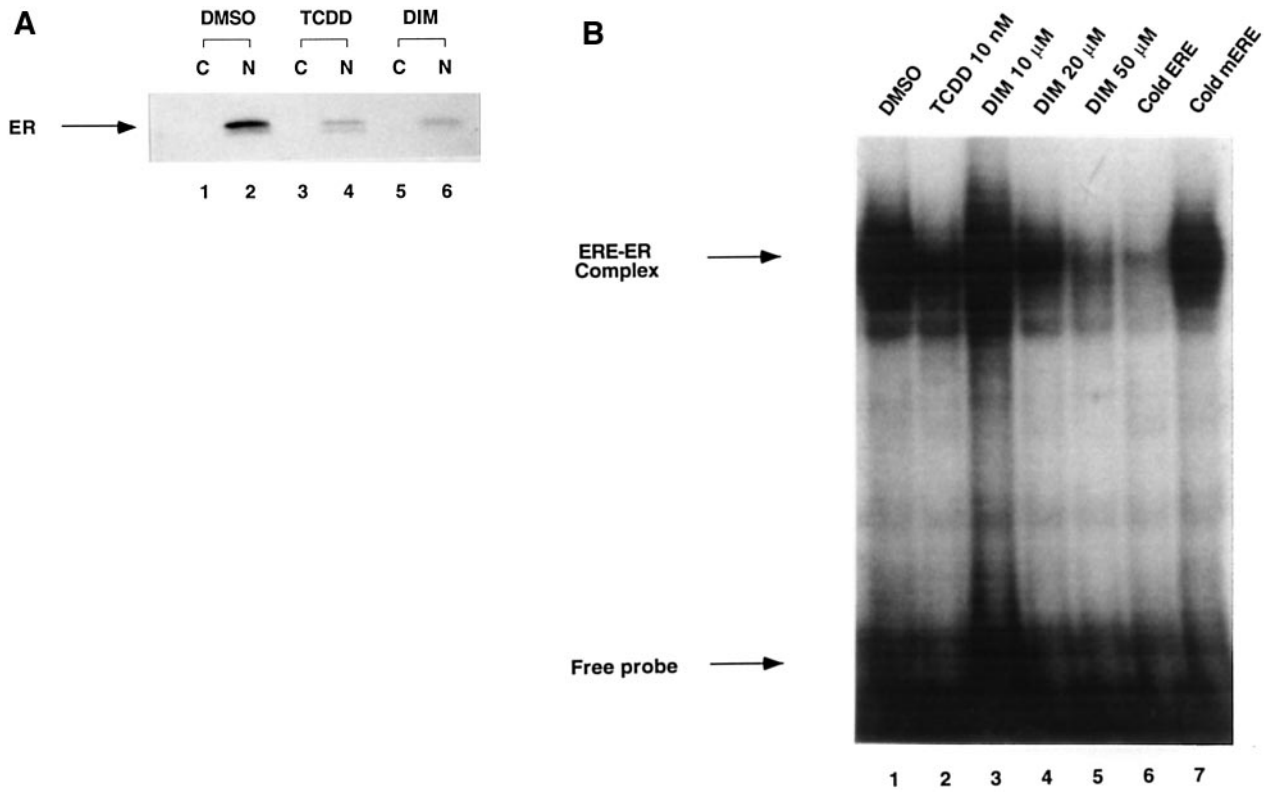

Fig. 5. Effect of DIM and TCDD on the ER in MCF-7 cells. (**A**) Immunoreactive nuclear ER protein. Immunoreactive ER protein from MCF-7 cell cytosolic and nuclear extracts treated with DMSO, 10 nM TCDD and 50 μM DIM was determined as described in Materials and methods. Treatment with TCDD or DIM markedly decreased nuclear ER protein. (**B**) Nuclear ER–ERE retarded band formation. Gel retardation analysis of the ERE–nuclear extract complexes derived from MCF-7 cells treated with DMSO, 10 nM TCDD or 10, 20 or 50 μM DIM for 12 h. Nuclear extracts were incubated with [^{32}P]ERE, separated by 5% PAGE and visualized by autoradiography as described in Materials and methods. Treatment with TCDD and DIM resulted in a decrease in formation of the specifically bound [^{32}P]ERE–ER complex. Relative intensities of the specifically bound [^{32}P]ERE–ER complex using nuclear extracts from MCF-7 cells treated with DMSO (arbitrarily set at 100), 10 nM TCDD and 10, 20 and 50 μM DIM (lanes 1–5 respectively) were 100 ± 11, 50 ± 9*, 81 ± 28, 42 ± 9* and 11 ± 2* respectively. The ERE–ER complex band from DMSO-treated MCF-7 cells was reduced in intensity after incubation with a 25-fold excess of unlabeled ERE (lane 6) but no decrease was observed after co-incubation with excess unlabeled mutant ERE (lane 7). *Significantly decreased ($P < 0.05$). Results are means ± SE for at least three replicate determinations.

days. At lower doses of DIM (1.0 and 0.5 mg/kg), no significant inhibition of mammary tumor growth was observed. Treatment with DIM (5.0, 1.0 or 0.5 mg/kg) did not affect body or organ weights and no significant induction of hepatic microsomal EROD activity was observed at any dose (Table I). Light microscopic examination of mammary tumors and other organs from rats treated with DIM did not show any significant ultrastructural changes and these results were similar to those observed for alternate-substituted PCDFs (39).

Discussion

Breast cancer is one of the leading causes of premature death in North American women and it is estimated that during their lifetime one in nine women will be diagnosed with this disease (44,45). Over 60% of primary mammary tumors in women are ER-positive and many of these patients respond to endocrine therapy with antiestrogens such as tamoxifen (46). Successful management of this disease requires development of multiple treatment strategies with both antiestrogenic and cytotoxic drugs. Several studies have demonstrated that TCDD, a potent AhR agonist, exhibits antiestrogenic and antitumorigenic activity via crosstalk between the AhR and ER signaling pathways (36,47,48). Moreover, alternate-substituted alkyl PCDFs have been characterized as relatively non-toxic AhR agonists which exhibit antiestrogenic activity in cell culture and

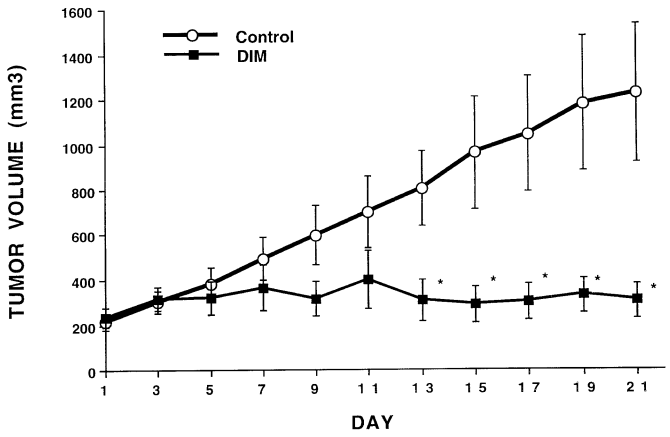

Fig. 6. Time course inhibition of mammary tumor growth by DIM (5 mg/kg). Animals were treated orally every other day with DIM or corn oil (control) and tumor volumes/mass were determined as described in Materials and methods.

antitumorigenic activity in the DMBA-induced rat mammary tumor model, thereby representing a group of compounds with potential for clinical treatment of breast cancer (39,49–51).

Previous studies have reported that DIM competitively binds to the AhR (33,35); however, at concentrations as high as

Table I. Dose-dependent antitumorigenic activity of DIM in DMBA-induced rat mammary tumors

Response	Experiment 1		Experiment 2		
	Control	DIM (5 mg/kg)	Control	DIM (1.0 mg/kg)	DIM (0.5 mg/kg)
No. of animals	10	9	8	10	9
Final tumor volume (mm^3)	1232 ± 309	305 ± 77[b]	1286 ± 504	1126 ± 346	1146 ± 455
Final tumor volume (g)	2.21 ± 0.68	0.63 ± 0.17[b]	1.74 ± 0.67	1.97 ± 0.63	1.88 ± 0.74
Final body wt (g)	238 ± 5.1	237 ± 3.8	261 ± 8	260 ± 4	263 ± 6
Hepatic EROD activity (pmol/min/mg)	150 ± 34	180 ± 32	248 ± 69	315 ± 172	278 ± 118
Liver/body wt (%)	3.86 ± 0.18	3.72 ± 0.20	3.37 ± 0.23	3.73 ± 0.14	3.35 ± 0.15

[a]Rats were dosed by gavage with 20 mg DMBA/rat on day 55. When the largest (or only) tumor in a dosed rat reached a size of 200–400 mm^3, rats were treated by gavage every other day with 5 mg/kg vehicle (corn oil) (in the first experiment) or 1.0 or 0.5 mg/kg DIM (in the second experiment). The experiments were terminated after 10 treatments, animals were killed and body and organ weights were routinely measured.
[b]Significantly different ($P < 0.05$) from control animals.

31 μM DIM only slightly induced CYP1A1 mRNA levels in T47D breast cancer cells (40). The results summarized in Figures 1 and 2 demonstrate the parallel effects of both TCDD and DIM on subcellular localization of the AhR in MCF-7 cells and induction of *CYP1A1* gene expression (Figures 1 and 2). Treatment of MCF-7 cells with 10 nM TCDD or 50 μM DIM for 1.5 h resulted in rapid cytosolic–nuclear translocation of the immunoreactive protein (Figure 1B) and this was confirmed in gel mobility shift and DRE crosslinking assays (Figure 2). These results confirm that DIM, like TCDD, activates the AhR complex, which is a ligand-induced transcription factor in MCF-7 cells, however, there were significant differences in the potency of these compounds, since TCDD was >10 000 times more active than DIM for induction of CYP1A1. Moreover, DIM induced formation of the nuclear AhR complex in gel mobility shift assays (Figure 2A) at a concentration (10 μM) which does not induce *CYP1A1* gene expression (Figure 1A). Similar results have previously been reported for alternate-substituted alkyl PCDFs, which also exhibit weak AhR agonist/partial antagonist activity for AhR-mediated CYP1A1 induction and other toxic responses (39,49–51).

Previous studies have reported that TCDD inhibits E2-induced proliferation of MCF-7 cells, down-regulates nuclear ER protein levels and inhibits hormone-induced transactivation of several E2-responsive genes or promoter–reporter constructs (reviewed in ref. 36). Comparable *in vitro* antiestrogenic responses were observed for TCDD and DIM in this study (Figures 3–5) and confirm that both compounds elicit AhR-mediated antiestrogenic responses. Ah-non-responsive MCF-7^{BaPr} cells express the ER (43) and show E2-induced proliferation. Although the AhR is expressed in MCF-7^{BaPr} cells, the nuclear or transformed cytosolic AhR complex does not bind DNA and these cells exhibit low Ah responsiveness (43). Only minimal inhibition of E2-induced proliferation of MCF-7^{BaPr} cells was observed for 5 μM DIM and at lower concentrations no antiestrogenic effects were observed (Figure 3B), whereas antiestrogenic activity was observed in wild-type Ah-responsive cells at concentrations as low as 0.1 μM (Figure 3A). These effects may be AhR-independent or be due to low level expression of a functional AhR. Interestingly, 5 μM DIM alone induced proliferation of mutant MCF-7^{BaPr} but not wild-type MCF-7 cells; the reason for this apparent ER-independent mitogenic response is unknown. MCF-7^{BaPr} cells are less estrogen-responsive than wild-type cells and it is possible that the mitogenic effects of 5 μM DIM may be estrogen independent. This is currently being investigated.

Induction of *CYP1A1* gene expression and antiestrogenic activities by TCDD are usually observed at comparable concentrations in MCF-7 cells. In contrast, DIM significantly inhibited E2-induced cell proliferation and CAT activity in cells transiently transfected with the Vit–CAT plasmid at concentrations of 0.1 and 10 μM, whereas 50–100 μM DIM induced *CYP1A1* gene expression. These results are similar to those previously reported for alternate-substituted alkyl PCDFs, such as 6-methyl-1,3,8-trichlorodibenzofuran (MCDF), which are relatively non-toxic and weak inducers of CYP1A1 but exhibit higher potencies as antiestrogens in MCF-7 cells and in the DMBA-induced rat mammary tumor model (39,49–51).

The results in Table 1 and Figure 6 show that administration of 5 mg/kg DIM every other day significantly inhibited DMBA-induced growth of mammary tumors in female Sprague–Dawley rats and this was not accompanied by induction of hepatic CYP1A1-dependent EROD activity, changes in organ/body weights or histopathology. At lower doses of DIM (1.0 and 0.5 mg/kg every 2 days), no antitumorigenic activity is observed. The *in vivo* results obtained with DIM are similar to those observed for MCDF, namely antitumorigenic activity at doses which do not induce EROD activity or alter organ weights or histopathology (39). Thus, it is unlikely that induction of drug-metabolizing activities by DIM plays a role in the observed antiestrogenic responses.

Previous studies with cruciferous vegetables, I3C and DIM have demonstrated their antitumorigenic activity in animal models for hormone-independent and -dependent tumors (14–23) and the treatment regimens were consistent with antitumorigenic activity associated with altered carcinogen metabolism. Previous studies on inhibition of estrogen-induced mammary tumors in rodents do not exclude an AhR-mediated antitumorigenic response; however, these studies were not designed to investigate this mode of action. In contrast, results reported in this paper demonstrate that DIM exhibits AhR agonist activity, inhibits multiple E2-induced responses in MCF-7 cells and inhibits DMBA-induced rat mammary tumor growth. Although the mechanisms of mammary tumor growth inhibition are unknown, we are currently investigating effects on specific cell cycle enzymes, since results of current ongoing studies in MCF-7 cells show that the AhR modulates several estrogen-induced cell cycle enzymes (A.McDougal, unpublished data). Thus, DIM represents a new class of relatively non-toxic antitumorigenic/antiestrogenic AhR agonists which are of phytochemical origin. Current research is focused on structure–function relationships for synthetic analogs of DIM and identi-

fying specific congeners for future use in clinical treatment of breast cancer.

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