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THE ARYL HYDROCARBON RECEPTOR AS A TARGET FOR ESTROGEN RECEPTOR-NEGATIVE BREAST CANCER CHEMOTHERAPY

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Abstract

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and the relatively non-toxic selective aryl hydrocarbon receptor (AhR) modulator (SAhRM) 6-methyl-1,3,8-trichlorodibenzofuran (MCDF) induced CYP1A1-dependent ethoxyresorufin *O*-deethylase (EROD) activity and inhibited proliferation of seven estrogen receptor (ER) negative breast cancer cell lines. MCDF, TCDD and structurally related 2,3,7,8-tetrachlorodibenzofuran (TCDF), 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin (PCDD), 2,3,4,7,8-pentachlorodibenzofuran (PCDF), and 3,3',4,4',5-pentachlorobiphenyl (PCB) induced CYP1A1 and inhibited proliferation of BT474 and MDA-MB-468 cells. In BT474 and MDA-MB-468 cells transfected with a small inhibitory RNA for the AhR (iAhR), the antiproliferative activity of the chlorinated aromatic compounds was reversed, whereas for MCDF, only partial reversal was observed, suggesting that this compound acts through both AhR-dependent and AhR-independent pathways in these two cell lines. MCDF also inhibited tumor growth in athymic nude mice in which MDA-MB-468 cells were injected directly into the mammary fat pad. These results suggests that the AhR is a potential drug target for treatment of ER-negative breast cancer.

Keywords

AhR agonists; chlorinated aromatics; SAhRMs; antiproliferative; ER-negative

INTRODUCTION

The AhR was initially identified as a receptor that bound the environmental toxicant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) with high affinity and studies with AhR knockout mice have confirmed a role for this protein in mediating TCDD-induced toxicity (Poland *et al.* 1976; Poland & Knutson 1982; Gonzalez & Fernandez-Salguero 1998; Schmidt *et al.* 1996). The mechanism of AhR action is similar to that described for other ligand-activated receptors and was determined in early studies on AhR-mediated induction of CYP1A1 gene expression

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[reviewed in (Whitlock *et al.* 1996; Whitlock, Jr. 1993)]. The unbound cytosolic AhR is associated with heat shock protein 90 (Hsp 90) and other factors and, in the presence of a ligand, the bound receptor forms a heterodimeric nuclear AhR complex containing the AhR and AhR nuclear translocator (Arnt) proteins. This complex binds dioxin response elements (DREs) in target gene promoters to induce transcriptional activation.

TCDD modulates an increasing number of biochemical, toxic and endocrine responses and research in the laboratory has focused on an intriguing AhR-mediated response, namely the tissue-specific inhibition of estrogen-induced genes and pathways (Safe & Wormke 2003; Safe 2005). Kociba and coworkers (Kociba *et al.* 1978) initially reported that dietary administration of TCDD to female Sprague Dawley rats inhibited age-dependent spontaneous mammary and uterine tumor formation. Subsequent studies in breast cancer cells and other E2-responsive tissues have characterized inhibitory AhR-ER crosstalk at the gene, response and mechanistic level and it is clear that multiple pathways are involved. For example, TCDD induces AhR-dependent degradation of ER via activation of proteasomes and this is due, in part, to the ubiquitin ligase activity of the AhR complex (Wormke *et al.* 2003; Ohtake *et al.* 2007).

Studies in several laboratories have demonstrated that the AhR may be a potential drug target for a number of diseases including ER-positive breast cancer, endometrial, prostate and pancreatic cancer and also for some autoimmune diseases (Koliopanus *et al.* 2002; McDougal *et al.* 1997; McDougal *et al.* 2001; Jana *et al.* 2000; Morrow *et al.* 2004; Castro-Rivera *et al.* 1999; Wormke *et al.* 2000; Quintana *et al.* 2008; Veldhoen *et al.* 2008; Kimura *et al.* 2008; Lawrence *et al.* 2008). Development of relatively non-toxic selective AhR modulators (SAhRMs) as drugs has been reported (Safe & McDougal 2002; Safe *et al.* 1999) and 6-methyl-1,3,8-trichlorodibenzofuran (MCDF) and other alternate-substituted dibenzofurans are highly effective agents for inhibiting hormone-responsive breast cancer growth in animal models (McDougal *et al.* 2001; Safe & McDougal 2002; Safe *et al.* 1999).

The AhR is also expressed in ER-negative breast cancer cells (Wang *et al.* 1997; Wang *et al.* 1995); however, the effectiveness of AhR agonists and SAhRMs against this highly aggressive form of late-stage breast cancer has not been extensively investigated. One report showed that TCDD inhibited ER-negative MDA-MB-468 cell proliferation and this was associated with induction of transforming growth factor- α (TGF α) which exhibits antiproliferative activity in this cell line (Wang *et al.* 1997). This study investigates the Ah-responsiveness of several different ER-negative breast cancer cell lines including MDA-MB-453, HCC-38, MDA-MB-436, MDA-MB-345, BT-474, MDA-MB-157 and MDA-MB-468 cells using the following AhR agonists: TCDD, 1,2,3,7,8-pentachlorodibenzo-p-dioxin (PCDD), 2,3,7,8-tetrachlorodibenzofuran (TCDF), 2,3,4,7,8-pentachlorodibenzofuran (PCDF), 3,3',4,4',5-pentachlorobiphenyl (PCB), and MCDF. These AhR agonists all induced CYP1A1-dependent activity and decreased proliferation of ER-negative breast cancer cell lines, and RNA interference studies with a small inhibitory RNA for the AhR (iAhR) confirmed that for TCDD and related chlorinated aromatics, their effects on cell growth were AhR-dependent. The effects of MCDF on breast cancer cell proliferation were both AhR-dependent and AhR-independent and this compound also inhibited tumor growth in athymic nude mice in which MDA-MB-468 cells were injected into the mammary fat pad.

MATERIALS AND METHODS

Cell lines, constructs, and antibodies

BT474, HCC-38, MDA-MB-453, MDA-MB-435, MDA-MB-436, MDA-MB-157, and MDA-MB-468 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The pDRE3-luciferase reporter plasmid was constructed in this laboratory and contained three tandem consensus dioxin response elements (DRE) (TCT TCT CAC GCA ACT CCG A

— a single DRE sequence). Antibodies for CYP1A1, AhR and Arnt proteins were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antibody for β -actin was obtained from Sigma-Aldrich (St. Louis, MO).

Ethoxyresorufin O-deethylase (EROD) activity

Trypsinized cells were plated into 25 cm² tissue culture flasks (10⁵ cells/ml), allowed to attain 60% confluency, and treated with 10 nM TCDD for 24 hr. Cells were harvested by manual scraping from the plate, centrifuged at 400 \times g for 5 min at 4°C and resuspended in 100 μ l Tris-sucrose buffer (38 mM Tris-HCl, 0.2 M sucrose; pH 8.0). Aliquots (50 μ M) of the cells were incubated with 1.15 ml cofactor solution (1 mg bovine serum albumin, 0.7 mg NADH, 0.7 mg NADPH, 1.5 mg MgSO₄ in 0.1 M HEPES buffer; pH 7.5) in a 37°C water bath for 2 min. The reaction was started by adding 50 μ l ethoxyresorufin solution (1 mg ethoxyresorufin/40 ml methanol). After incubation for 15 min, the reaction was stopped by adding 2.5 ml methanol. Samples were centrifuged for 10 min at 1500 \times g. The supernatant was analyzed by fluorescence measurement at an excitation wavelength of 550 nm, and an emission wavelength of 595 nm.

Transient transfection assays

Cells were cultured in 12-well plates in 1 ml of DME/F12 medium supplemented with 2.5% fetal bovine serum. After 16–20 hr when cells were 30–50% confluent, the pDRE-luc (0.4 μ g) and β -galactosidase (0.1 μ g) constructs were transfected using Lipfectamine 2000 Reagent (Invitrogen, Carlsbad, CA) and after 12 hr, cells were treated with DMSO or the AhR agonists. Cells were harvested 36–44 hr after transfection by manual scraping in 1 \times lysis buffer (Promega, Madison, WI). For whole cell lysates, cells were frozen and thawed in liquid nitrogen, vortexed for 30 s, and centrifuged at 12,000 \times g for 1 min. Lysates were assayed for luciferase activity using luciferase assay reagent (Promega). β -Galactosidase activity was measured using Tropix Galacto-Light Plus assay system (Tropix, Bedford, MA) in a Lumicount microwell plate reader (Packard Instrument Co.).

Western immunoblot assay

Cells were seeded into 35-mm six-well tissue culture plates in phenol red-free DME/F12 medium supplemented with 2.5% dextran/charcoal-stripped fetal bovine serum. After 24 hr, cells were treated with the five AhR agonists or DMSO (solvent control) for 24 hr and harvested in ice-cold high salt lysis buffer (50 mM HEPES, 500 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM EGTA, pH 7.5) supplemented with protease inhibitor cocktail (Sigma). An aliquot of the whole cell lysates containing 30 μ g protein was diluted with loading buffer, boiled, and loaded on a 10% SDS-polyacrylamide gel. Samples were electrophoresed at 150–180 V for 3–4 hr and separated proteins were transferred to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). Proteins were detected by incubation with polyclonal primary antibodies against CYP1A1, AhR, Arnt or β -actin (1:1000 dilution), followed by blotting with horseradish peroxidase-conjugated anti-rabbit (for CYP1A1, AhR and Arnt) or anti-mouse (for β -actin) secondary antibody (1:5000 dilution).

Cell proliferation and fluorescence-activated cell sorting (FACS)

Cells were transfected with iAhR or scrambled oligonucleotide. Thirty-six hr after the transfection, cells were trypsinized, syringed and collected by centrifugation. Cells were resuspended in staining solution [50 μ g/mL propidium iodide, 30 units/mL RNase, 4 mmol/L sodium citrate, and Triton X-100 (pH 7.8)] and incubated at 37°C for 10 min. Sodium chloride solution was added to a final concentration of 0.15 mol/L. Stained cells were analyzed on a FACS Calibur Flow Cytometer (Becton Dickinson Immunocytometry Systems) using Cell Quest (Becton Dickinson Immunocytometry Systems) acquisition software. For cell

proliferation studies, cells were transfected with iAhR or scrambled oligonucleotide using Lipofectamine 2000 reagent (Invitrogen); the medium was changed after 5 hr, and 4 or 7 days, later cells were counted using a Coulter Z1 cell counter (Beckman Coulter).

RNA interference studies

The siRNA targeting AhR was purchased from Dharmacon (Lafayette, CO), with the sequences of: 5'-UAA GGU GUC UGC UGG AUA AUU -3'. The nonspecific siRNA (4613) was purchased from Ambion (Austin, TX) as a negative control. Before the transfection process, cells were seeded in 12 well-plates in DME/F12 medium (Sigma-Aldrich) supplemented with 2.5% dextran/charcoal-stripped fetal bovine serum. After 24 hr, appropriate amounts of plasmids and/or siRNA duplexes were transfected using lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to manufacturer's recommendations. After 6–8 hr, cells were changed to fresh medium and appropriate chemical treatments were added.

In vivo studies with MCDF

Athymic nude Hsd:nu/nu homozygous female virgin mice were purchased from Harlan (Houston, TX) at 3–4 weeks of age and were transported and maintained under sterile conditions. Cancer cells were grown to 90% confluency, trypsinized, centrifuged and resuspended in 200 μ l of a 1:1 solution of PBS plus Matrigel (Collaborative Biomed, Bedford, MA) at 4°C. Mice (5 animals per treatment group) were injected subcutaneously in both mammary fat pads, with 0.7×10^7 cells/site in a matrigel suspension. After approximately 7 days, mice were treated with corn oil (vehicle control) or MCDF (25 mg/kg) in corn oil by gavage every second day, and tumors were measured with a micrometer. Tumor area was calculated by the equation: Area = (length/2) \times (width/2) \times π . Statistical differences were determined as indicated below or by the student's t-test and significant ($p < 0.05$) differences using these test were consistently observed from days 16 – 22.

Statistical analysis

Statistical significance was determined by analysis of variance and Scheffe's test, and the levels of probability are noted. The results are expressed as means \pm SE for at least three separate (replicate) experiments for each treatment group in the *in vitro* studies.

RESULTS

Previous studies show that inhibitory AhR-ER α crosstalk in breast cancer cells results in inhibition of E2-induced growth and gene expression in ER-positive breast cancer cells (Safe & Wormke 2003); however, the Ah-responsiveness and growth inhibitory effects of AhR agonists in ER-negative breast cancer cells is not well defined. Therefore, we initially investigated the Ah-responsiveness of several ER-negative breast cancer cells by determining the effects of TCDD on the induction of CYP1A1-dependent EROD activity. TCDD significantly induced EROD activity in two cell lines that overexpress the oncogene ErbB2 (BT474 and MDA-MB-453 cells) and also significantly induced this response in MDA-MB-435, HCC-38, MDA-MB-157, and MDA-MB-436 cells (Fig. 1). The dose-response curves and fold-inducibility were highly variable; however significant induction of EROD activity was observed in all cell lines. These results coupled with previous studies in MDA-MB-468 cells show that ER-negative breast cancer cells are Ah-responsive (Wang *et al.* 1997). The BT474 cells used in this study did not express ER α and this is illustrated in supplement Figure 1.

In addition, we also investigated the growth inhibitory effects of TCDD on this same group of ER-negative breast cancer cells (Fig. 2). Incubation of these cells with TCDD significantly decreased cell proliferation after treatment for 4 or 6 days. Two ER-negative lines, BT20 and

MDA-MB-134, exhibited minimal Ah-responsiveness (induction of EROD activity by TCDD) and we also observed that TCDD did not appreciably inhibit growth of these cell lines (data not shown).

Previous studies indicate that the selective AhR modulator, MCDF, inhibits ER-positive breast cancer cell and tumor growth *in vivo* (McDougal *et al.* 2001), and this compound exhibits low toxicity and minimal induction of AhR-mediated toxic responses (Astroff *et al.* 1988; Harris *et al.* 1989; Bannister *et al.* 1989; Yao & Safe 1989). We also investigated the effects of MCDF on proliferation of ER-negative breast cancer cells. Results (Fig. 3) indicate that MCDF also inhibited growth of ER-negative breast cancer cells; however, this was accompanied by variable induction of CYP1A1-dependent EROD activity (data not shown) as previously observed in ER-positive breast cancer cells (Safe & McDougal 2002; Safe *et al.* 1999).

These data suggest that AhR ligands such as TCDD and MCDF decrease proliferation of ER-negative breast cancer cell lines; however, with the exception of previous studies with TCDD in MDA-MB-468 cells (Wang *et al.* 1997), the expression and role of the AhR in mediating the growth inhibitory effects of AhR agonists in ER-negative breast cancer cells has not been determined. In this study, we used TCDD and related chlorinated aromatics with known differences in their potencies as AhR agonists (Van den Berg *et al.* 2006). Figures 4A and 4B show that TCDD and related chlorinated aromatics and MCDF induced luciferase activity in BT474 and MDA-MB-468 cells transfected with an Ah-responsive DRE-luc construct, and treatment of these cells with the same compounds also resulted in the induction of CYP1A1 protein; in addition, the AhR was also expressed in both cell lines (Fig. 4C) and ErbB2 was highly expressed in BT474 cells but only minimal expression was observed in MDA-MB-468 cells (data not shown). The effects of these compounds on cell growth were also investigated in BT474 and MDA-MB-468 cells and the results (Fig. 4D) indicate that at the concentrations used in this study, all of the congeners significantly decreased proliferation of BT474 and MDA-MB-468 cells. Higher concentrations of AhR agonists were used in this 96 hr cell proliferation study compared to the 6 day experiments (Fig. 2 and Fig. 3) to ensure significant growth inhibition.

The role of the AhR in mediating the effects of the AhR agonists on ER-negative breast cancer cell survival was also investigated in BT474 and MDA-MB-468 cells transfected with a non-specific oligonucleotide (iCtr) and a small inhibitory RNA for the AhR (iAhR). Data in Figure 5A show that transfection with iAhR resulted in a > 80% decrease in AhR expression in BT474 and MDA-MB-468 cells. Knockdown of the AhR in BT474 cells resulted in a significant increase in cell proliferation compared to cells transfected with iCtr (Fig. 5B). This indicated that in BT474 cells, basal expression of the AhR inhibited cell proliferation, and similar results were previously reported in ER-positive MCF-7 breast cancer cells (Abdelrahim *et al.* 2003). In contrast, a comparison of cell numbers in MDA-MB-468 cells transfected with iCtr or iAhR treated with DMSO indicated that basal expression of the AhR did not affect proliferation of this cell line (Fig. 5B). The effects of AhR knockdown on distribution of BT474 and MDA-MB-468 cells in G₀/G₁, S and G₂/M phases of the cell cycle were also determined (Fig. 5C). No significant effects were observed in BT474 cells, whereas AhR knockdown in MDA-MB-468 cells decreased cells in G₀/G₁ and induced a G₂/M arrest.

Due to the temporal limitations in AhR knockdown by RNA interference, we used higher concentrations of AhR agonists in the short term inhibition of cell proliferation study summarized in Figures 6A and 6B. Treatment of BT474 cells with 5 μM MCDF, 40 μM TCDD, 40 μM PCDD, 40 μM PCDF, 40 μM TCDF and 100 μM PCB all significantly decreased BT474 cell proliferation. In contrast, after transfection with iAhR, the antiproliferative effects of the AhR agonists were significantly inhibited and the chlorinated aromatics (TCDD, PCDD, TCDF, PCDF and PCB) did not significantly inhibit growth of BT474 cells compared to the

solvent (DMSO) control. MCDF partially inhibited BT474 cell growth, even after AhR knockdown, suggesting that the growth inhibitory effects of this compound were both AhR-dependent and AhR-independent. The effects of iAhR on MDA-MBA-468 cell proliferation after treatment with the same set of compounds showed that the AhR agonist-dependent inhibition of growth was blocked after AhR knockdown by RNA interference (Fig. 6B). Moreover, the results obtained for MCDF were similar in MDA-MB-468 and BT474 cells, indicating an AhR-dependent and AhR-independent mechanism of action for this compound in both cell lines. Results in Figure 6C demonstrate that MCDF (25 mg/kg every second day) also inhibited growth of tumors in athymic nude mice bearing MDA-MB-468 cells injected directly into the mammary fat pad. Tumors derived from MDA-MB-468 cells grew slowly and consistent differences in tumor area between control and MCDF treatment groups were not observed until day 16 and significant ($p < 0.05$) differences were observed from days 16 – 22. Treatment with MCDF did not significantly affect body, liver, uterine, heart, spleen or kidney weight or expression of hepatic CYP1A1 (data not shown). These results demonstrate the potential clinical applications of SAhRMs for treatment of ER-negative breast cancer.

DISCUSSION

Breast cancer is a highly complex disease in which treatment options depend on the staging of the tumor, localization or spreading of the tumor, and the molecular characteristics of the tumor including its estrogen receptor status or expression of other genes such as the ErbB2 (HER2/neu) oncogene (Moulder & Hortobagyi 2008; Buzdar 2003; Macaskill & Dixon 2007). Many early stage mammary tumors are ER-positive and have been successfully treated with antiestrogens such as tamoxifen, raloxifene, fulvestrant or aromatase inhibitors (Fisher *et al.* 2005; Vogel *et al.* 2006; Semiglazov *et al.* 2007; Howell *et al.* 2005). Prolonged use of tamoxifen can result in development of drug-resistant tumors and there is evidence that long term use of tamoxifen increases the risk for endometrial cancer (Vogel *et al.* 2006; Clarke *et al.* 2001). Some early stage and most later stage mammary tumors are ER-negative and patients with ER-negative breast cancer do not respond well to endocrine therapy and successful adjuvant chemotherapy requires the use of more highly cytotoxic drugs commonly used to treat other endocrine-independent tumors (Semiglazov *et al.* 2007; Moulder & Hortobagyi 2008). These agents generally target some aspect of nuclear function or modulate microtubule formation/breakdown and include compounds such as adriamycin, cyclophosphamide, gemcitabine, taxanes (taxol and taxotere), and capecitabine, a precursor of 5-FU (Moulder & Hortobagyi 2008). More recently, there has been an increase in the applications and development of more targeted therapies that include antibodies that interact with the angiogenic factor vascular endothelial growth factor (VEGF). In addition, tyrosine kinase inhibitors that target VEGF receptor and growth factor receptors have also been developed for clinical treatment of breast cancer (Moulder & Hortobagyi 2008; Buzdar 2003; Macaskill & Dixon 2007; Hobday & Perez 2005; Demonty *et al.* 2007). Another important advance for breast cancer treatment has been the increased use of combined agents which often target different pathways responsible for tumor survival, growth, angiogenesis and metastasis. Herceptin or trastuzumab is a monoclonal antibody directed against the extracellular domain of ErbB2 and objective response rates of 25–40% are observed with this antibody in patients that overexpress ErbB2 (Demonty *et al.* 2007).

Drugs such as MCDF that target the AhR are highly effective for inhibition of E2-responsive tumor growth in carcinogen-induced female Sprague Dawley rats, and MCDF and tamoxifen in combination synergistically blocked tumor formation and growth (McDougal *et al.* 2001). Although the AhR is widely expressed in ER-negative and ER-positive breast cancer cell lines (Wang *et al.* 1997; Wang *et al.* 1995), the potential applications of AhR agonists for treatment of ER-negative breast cancer is not well defined. One study in ER-negative MDA-MB-468 cells showed that TCDD inhibited survival of these cells through the induction of TGF α which

exhibits antiproliferative activity in this cell line (Wang *et al.* 1997). Figure 1 shows that in addition to MDA-MB-468 cells, at least six other ER-negative breast cancer cell lines including two that overexpress ErbB2 (BT474 and MDA-MB-468 cells) were Ah-responsive, and TCDD and five structurally related chlorinated aromatics induced CYP1A1-dependent EROD activity. In this study, TCDD did not induce EROD activity in BT20 and MDA-MB-134 cells, and the reasons for the lack of Ah-responsiveness in these cell lines are currently being investigated. We also examined the comparative effects of TCDD and MCDF on survival of this panel of ER-negative breast cancer cells (Fig. 2 and Fig. 3) and both compounds significantly decreased growth of the six Ah-responsive cell lines.

The AhR interacts with structurally diverse ligands including synthetic aromatics, phytochemicals such as flavonoids and indole derivatives, drugs, pesticides, endogenous biochemicals including bilirubin, and other polyaromatics (Denison & Nagy 2003). The structure-dependent potencies of chlorinated aromatics such as TCDD, TCDF, PCDF, PCDD and PCBs as AhR agonists has been extensively investigated (Van den Berg *et al.* 2006) and for some responses such as induction of CYP1A1, there is a rank order correlation between their structure-AhR binding versus structure-activity relationships. Results in Figures 4A and 4B show that the chlorinated aromatics and MCDF induced luciferase activity in BT474 and MDA-MB-468 cells transfected with an Ah-responsive DRE-luciferase construct. Moreover, treatment of the two cell lines with the same set of compounds also induced CYP1A1 protein and western blot analysis of whole cell lysates also showed that the AhR was expressed in BT474 and MDA-MB-468 cells (Fig. 4C). In previous studies with ErbB2-overexpressing BT474 and MDA-MB-453 cells, we also showed that TCDD and MCDF inhibited cell proliferation but did not affect ErbB2 or its phosphorylation, and downstream kinases were also unchanged (unpublished results). However, results of the CYP1A1 induction studies coupled with the structure-dependent effects of the chlorinated aromatics and MCDF on decreased BT474 and MDA-MB-468 cell proliferation (Fig. 4D) are consistent with a role for the AhR in mediating the effects of these compounds.

Endogenous expression of the AhR in cancer cell lines can affect cell growth (Abdelrahim *et al.* 2003). Knockdown of the AhR in ER-positive MCF-7 breast cancer cells enhanced cell proliferation, whereas in HepG2 liver cancer cells, AhR knockdown decreased the rate of cell growth (Abdelrahim *et al.* 2003). In this study, iAhR transfection in BT474 cells resulted in enhanced growth; however, this was not accompanied by changes in the % distribution of cells in G₀/G₁, S or G₂/M phases (Fig. 5C). Moreover, AhR agonists did not affect expression of ErbB2, phospho-ErbB2 or downstream kinases (data not shown), and we are currently investigating how the AhR and AhR agonists inhibit BT474 cell proliferation without changing the distribution of cells in G₀/G₁, S and G₂/M phases of the cell cycle. In contrast to BT474 cells, no significant changes in proliferation were observed in MDA-MB-468 cells transfected with iAhR (Fig. 5B) but these cells exhibited a decrease in G₀/G₁ and an arrest at G₂/M. Thus, the AhR differentially affects proliferation and % distribution of ER-negative breast cancer cells in G₀/G₁, S or G₂/M phases of the cycle, and current studies are investigating the cell context-dependent modulation of Ah-responsive genes, proteins and microRNAs that determine these responses. The growth inhibitory effects of the chlorinated aromatic compounds in BT474 and MDA-MB-468 cells (Fig. 4 and Fig. 6) were reversed in both cell lines after transfection with iAhR (Figs. 6A and 6B) and this was consistent with the role of the ligand-activated AhR in mediating the decreased proliferation of ER-negative breast cancer cell.

MCDF also decreased breast cancer cell survival and inhibited tumor growth in athymic nude mice bearing MDA-MB-468 cells as xenografts (Fig. 6). These data complement previous studies showing the effectiveness of this compound as a mammary tumor growth inhibitor in carcinogen-induced female Sprague-Dawley rats (McDougal *et al.* 2001). MCDF was initially

characterized as an AhR antagonist (McDougal *et al.* 2001) and studies with ^{125}I -MCDF showed that this compound bound the AhR and induced formation of a nuclear AhR complex in cancer cells (Piskorska-Pliszczynska *et al.* 1991). However, results of RNA interference studies with iAhR (Figs. 6A and 6B) demonstrate that loss of the AhR only partially reversed the antiproliferative effects of MCDF on BT474 and MDA-MB-468 cells. Thus, the anticancer activity of MCDF in ER-negative breast cancer cells is both AhR-dependent and AhR-independent, and current studies are focused on the molecular mechanisms associated with both pathways and application of MCDF and other SAhRMs for treatment of ER-negative breast cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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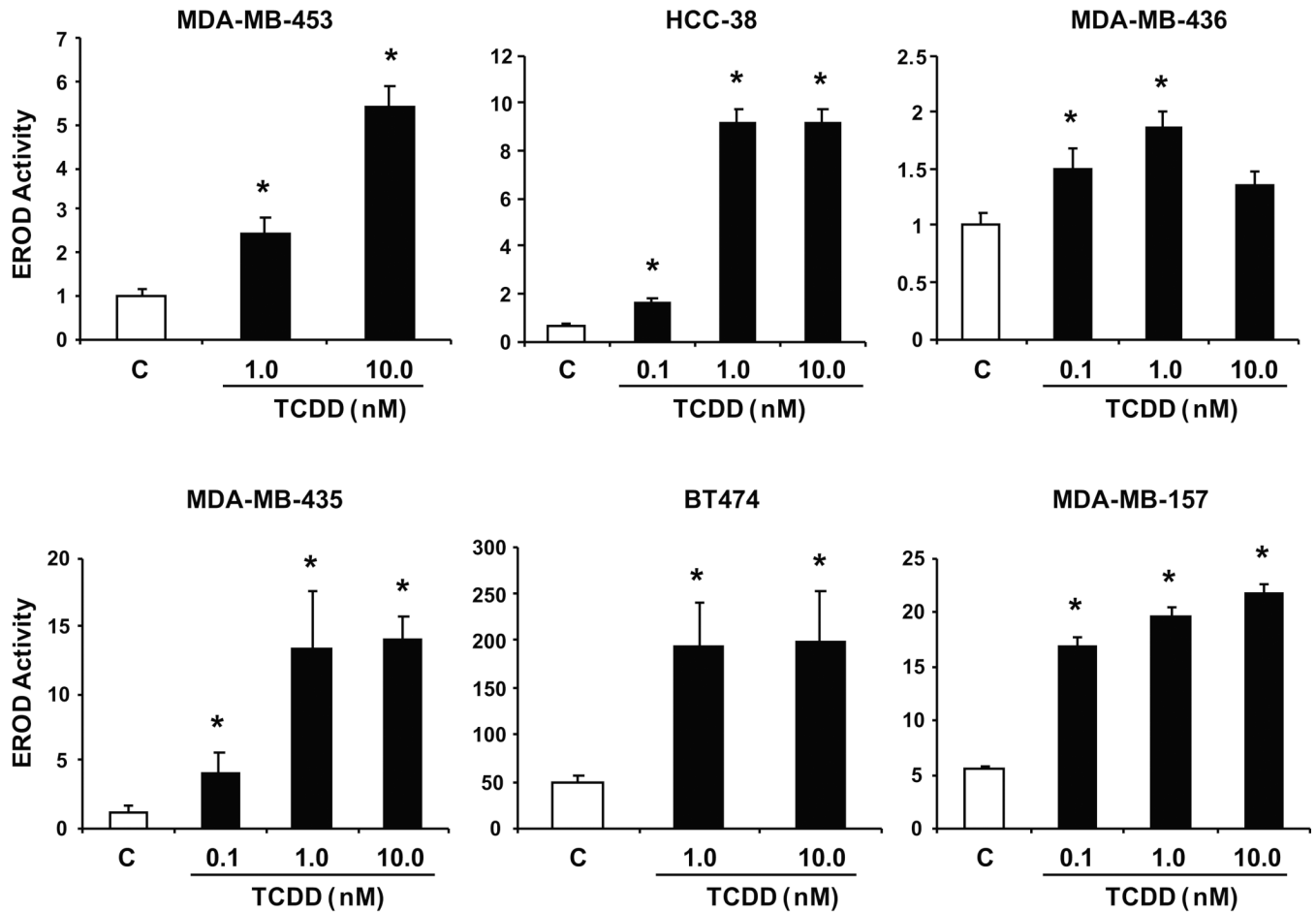


Figure 1.

Ah-responsiveness of ER-negative breast cancer cells. Induction of EROD activity by TCDD. ER-negative breast cancer cells were treated with DMSO or different concentrations of TCDD and EROD activity was determined as described in the Materials and Methods. Results are expressed as means \pm SE for 3 replicate determinations for each treatment group and significant ($p < 0.05$) induction is indicated (*).

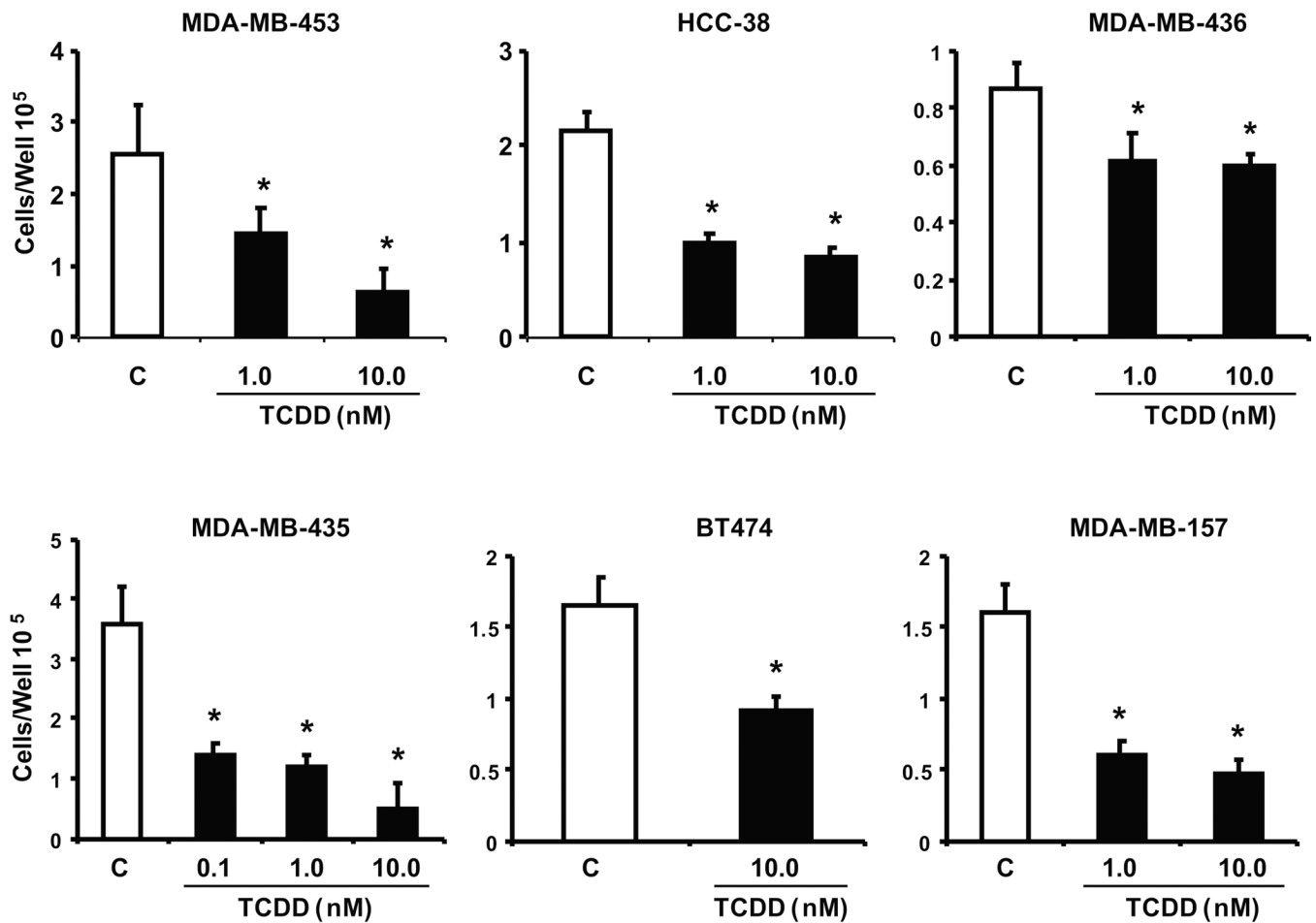


Figure 2. Antiproliferative activity of TCDD. Inhibition of ER-negative breast cancer cell growth. ER-negative breast cancer cell lines were treated with DMSO or different concentrations of TCDD for six days and cells were counted as described in the Materials and Methods. Results are expressed as means \pm SE for at least 3 replicate determinations for each treatment group and significant ($p < 0.05$) inhibition is indicated (*).

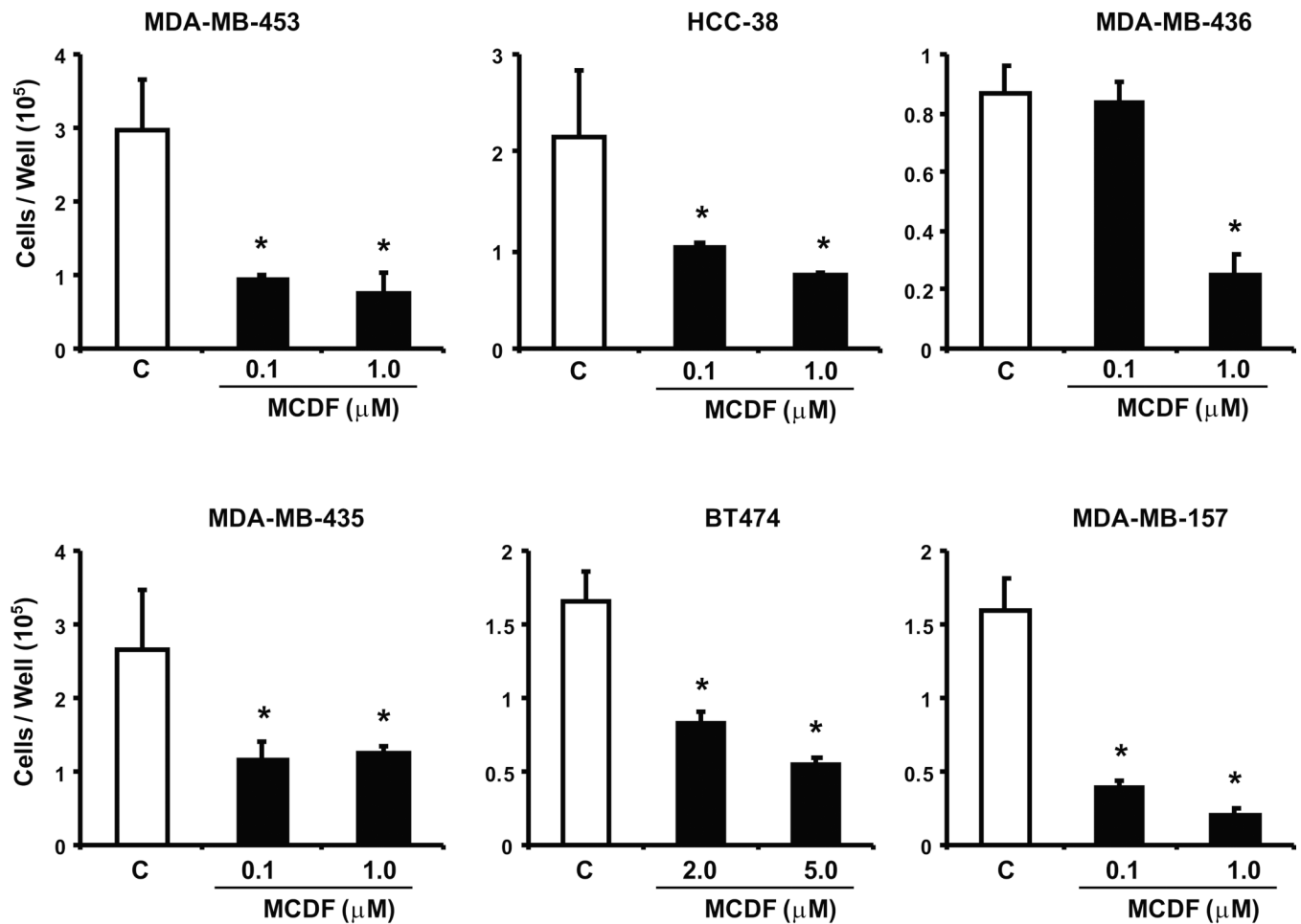


Figure 3.

Antiproliferative activity of MCDF. Inhibition of ER-negative breast cancer cell growth. ER-negative breast cancer cell lines were treated with DMSO or different concentrations of MCDF for six days and cells were counted as described in the Materials and Methods. Results are expressed as means \pm SE for 3 replicate determinations for each treatment group and significant ($p < 0.05$) of cell proliferation is indicated (*).

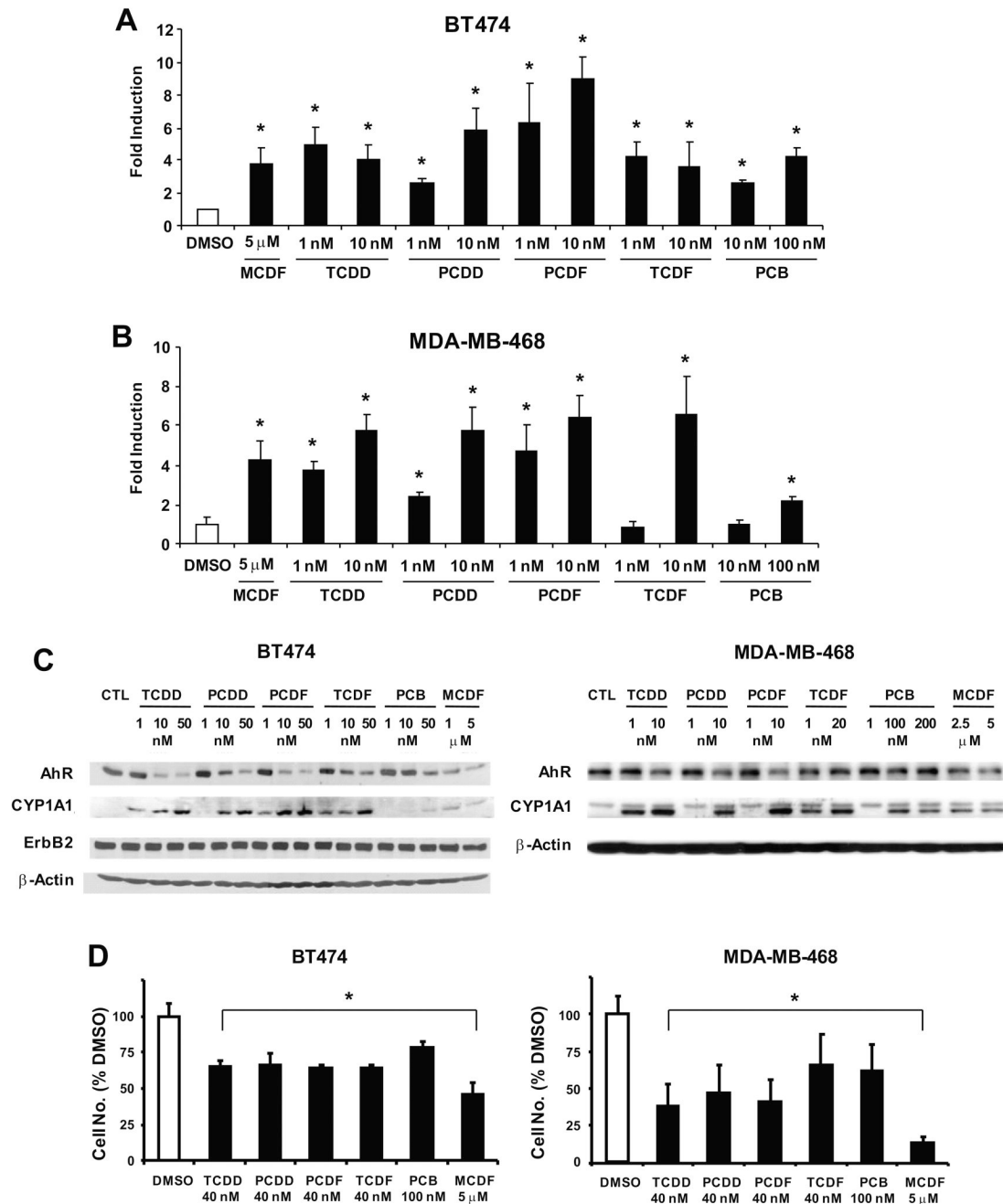


Figure 4. Structure-dependent activation of AhR-dependent responses by chlorinated aromatics in BT474 and MDA-MB-468 cells. Activation of DRE-luc in BT474 (A) and MDA-MB-468 cells (B). Cells were transfected with the DRE-luc construct and treated with DMSO or different concentrations of TCDD, PCDD, TCDF, PCDF and PCB, and luciferase activity determined as described in the Materials and Methods. Results are expressed as means \pm SE for 3 replicate determinations for each treatment group and significant ($p < 0.05$) induction is indicated (*). Structure-dependent induction of CYP1A1 protein and AhR expression (C) and growth inhibition (D) by AhR agonists. Cells were treated for either 24 (C) or 96 hr (D) and whole cell lysates were analyzed by western blots (C) or cells were counted (D) as described

in the Materials and Methods. Results in (D) are presented as means \pm SE for at least 3 replicate determinations for each treatment group and significant ($p < 0.05$) growth inhibition is indicated (*).

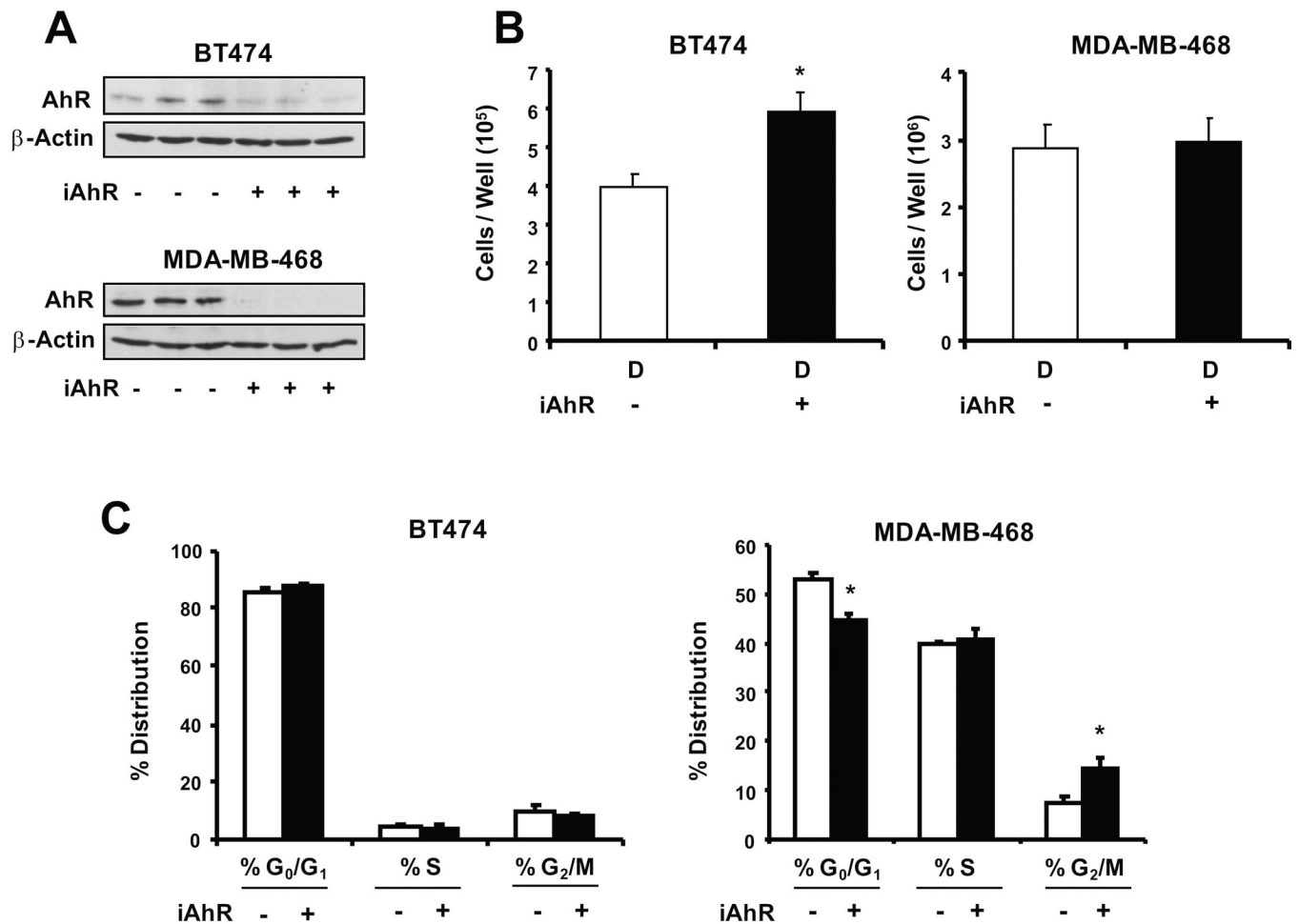
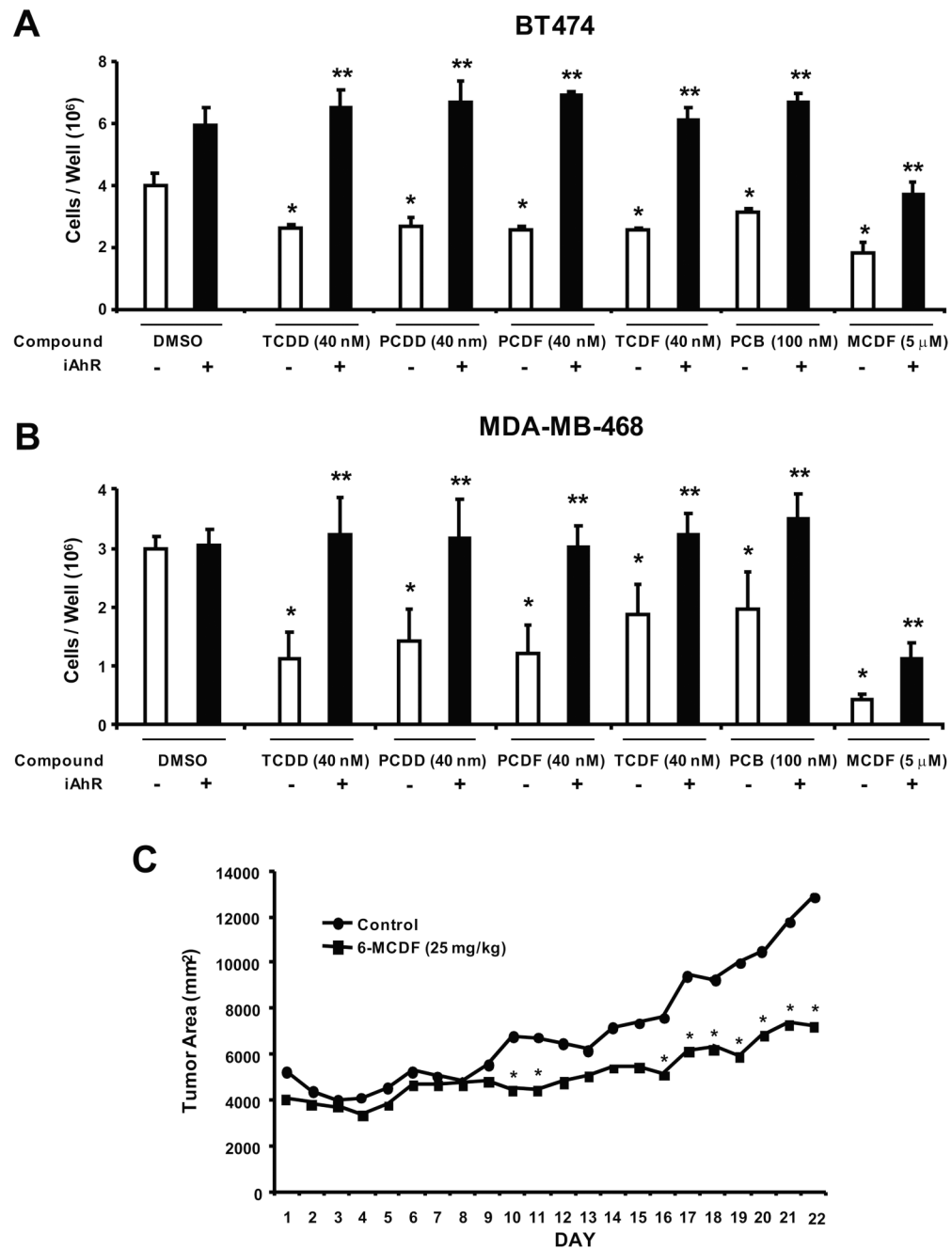


Figure 5. RNA interference and FACS analysis. Effects of iAhR on AhR protein (A) and cell proliferation (B). Cells were transfected with iAhR or non-specific oligonucleotide and the effects on AhR protein and proliferation of BT474 and MDA-MB-468 cells were determined as described in the Materials and Methods. Replicate (3) experiments were carried out for each treatment and, for the cell proliferation studies, results are expressed as means \pm SE (after treatment for 96 hr) and significant ($p < 0.05$) effects of iAhR on cell proliferation are indicated (*). (C) FACS analysis. The effects of iAhR on distribution of BT474 and MDA-MB-468 cells in G₀/G₁, S and G₂/M phases of the cell cycle were determined by FACS analysis as described in the Materials and Methods. Results obtained in cells transfected with iAhR are compared to cells transfected with a non-specific oligonucleotide as indicated above in (A) and (B). Results are expressed as means \pm SE for three replicate experiments and significant changes after transfection with iAhR are indicated (*).

**Figure 6.**

Antiproliferative and antitumorigenic activity of AhR agonists. Role of the AhR in mediating the antiproliferative effects of AhR agonists in BT474 (A) and MDA-MB-468 (B) cells. Cells were transfected with non-specific scrambled oligonucleotide (iCtr) or iAhR and treated with DMSO or different AhR agonists for 4 days, and the number of cells were counted as described in the Materials and Methods. Results are expressed as means \pm SE for at least 3 replicate determinations for each treatment group and significant ($p < 0.05$) inhibition of cell growth by the AhR agonists (*) and reversal of this effect by iAhR (**) are indicated. (C) Tumor growth inhibition. MDA-MB-468 cells were injected into the mammary fat pad of athymic nude mice and after palpable tumors were detected (7 – 10 days), mice were treated with corn oil (vehicle

control) or MCDF (25 mg/kg) every 48 hr. Tumor volumes were determined as described in the Materials and Methods. Significant ($p < 0.05$) inhibition of tumor growth is indicated by an asterisk. Body weights and liver, uterine, heart, spleen and kidney weights as % body weight in control/MCDF-treated mice were $26 \pm 1/25 \pm 1$, $5.2 \pm 0.3/5.3 \pm 0.1$, $0.35 \pm 0.1/0.35 \pm 0.04$, $0.46 \pm 0.01/0.49 \pm 0.01$, $0.72 \pm 0.1/0.66 \pm 0.01$ and $0.68 \pm 0.03/0.63 \pm 0.02$, respectively.