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14. ABSTRACT: Treatment for prostate cancer depends on multiple factors including the stage of the tumor and expression of the androgen receptor (AR). Endocrine therapy can be used for treatment of early stage androgen-responsive tumors, whereas chemotherapy for later stage androgen non responsive tumors is problematic. We investigated the aryl hydrocarbon receptor (AhR) as a potential target for treating prostate cancer using a new series of relatively non-toxic selective AhR modulators (SAhRMs). Initial studies showed that 22RV1, PC3 and LNCaP prostate cancer are Ah-responsive. Two SAhRMs, namely diindolylmethane (DIM) and 6-methyl-1,3,8- trichlorodibenzofuran (6-MCDF), inhibited growth of AR-positive 22RV1 and AR-negative PC3 cells. AhR ligands inhibited dihydrotestosterone-induced upregulation of AR protein in 22RV1 cells, suggesting a possible mechanism for inhibitory AhR-AR crosstalk. The growth inhibitory effects of SAhRMs in PC3 cells suggests that AhR ligands also inhibit growth of androgen-nonresponsive cells. In addition, substituted DIMs inhibit growth of prostate cancer cells and modulate AR expression, and these are currently being investigated.					
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INTRODUCTION

Prostate cancer is the most commonly diagnosed cancer in North American men and it is estimated that there are over 300,000 newly diagnosed cases each year (1, 2). The incidence and mortality rates from prostate cancer are increasing and this is due, in part, to an increasingly aging population and the higher incidence of this disease in older men (3, 4). Prostate cancer therapy is dependent on the stage of the tumor and AR expression. Early stage androgen-responsive prostate cancers can be treated by castration or with antiandrogens or drugs that block androgen-induced responses including steroidal antiandrogens (cyproterone), LHRH analogs, nonsteroidal antiandrogens (flutamide, nilutamide, bicalutamide), and the potent estrogenic drug diethylstilbestrol [reviewed in (5-8)]. In addition, there are several novel strategies for treatment of prostate cancer and other tumor-types and these include targeting of critical genes involved in tumor cell growth and metastasis (e.g. antiangiogenic drugs, antisense therapy) (9-13). Ligands for nuclear receptors (NR) are also being developed for treatment of prostate cancer through inhibitory NR-AR crosstalk that involves various compounds that bind the retinoid acid/X-receptors (retinoids), vitamin D receptor (calcitrol), and peroxisome proliferator activate receptor γ (troglitazone) (14-26). A recent study in androgen-responsive LNCaP prostate cancer cells showed that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a ligand for the aryl hydrocarbon receptor (AhR), inhibited testosterone-induced cell proliferation and gene/reporter gene expression (27). We have developed a series of alternate-substituted (2,3,6,8- and 2,4,6,8-) alkyl polychlorinated dibenzofurans (PCDFs) and substituted diindolylmethanes (DIMs) (Fig. 1) that inhibit rodent mammary tumor growth *in vivo*, but do not induce toxic responses associated with exposures to TCDD (28-35). These selective AhR modulators (SAhRMs) are therefore an important new class of drugs that target the AhR and they have been successfully used for

inhibiting growth of breast tumors/cells (28-35) and pancreatic cancer cells (36). Studies sponsored by this grant are focused on inhibitory AhR-AR crosstalk in human prostate cancer cells and applications of SAhRMs for treatment of this disease. These studies will include characterization of the Ah-responsiveness of several prostate cancer cells, inhibition of prostate cancer cell growth by SAhRMs, mechanisms of inhibitory AhR-AR crosstalk and *in vivo* inhibition of prostate tumor growth by SAhRMs in athymic nude mice bearing prostate cancer cell xenografts.

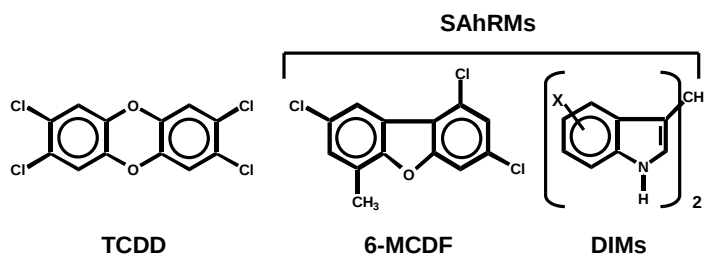


Figure 1. TCDD and selective AhR modulators (SAhRMs) 6-MCDF and DIM.

BODY

Characterization of Ah-responsiveness of prostate cancer cells

The first part of this project (Task 1) has been focusing on characterization of Ah-responsiveness of several prostate cancer cell lines by determining induction of CYP1A1-dependent activities by TCDD and by SAhRMs. The results in Figure 2 summarize the concentration-dependent induction of ethoxyresorufin O-deethylase (EROD) activity by TCDD (note: this activity is catalyzed by CYP1A1). TCDD clearly induces EROD activity in 22RV1 prostate cancer cells at concentrations of 0.1, 1.0 and 10.0 nM (Fig. 2A); the EC₅₀ value is < 0.1 nM and a > 14-fold induction response was observed. In a parallel experiment, DIM exhibited minimal induction of EROD activity (Fig. 2B), and this is consistent with previous reports

showing that DIM is a weak AhR agonist/partial antagonist for this response.

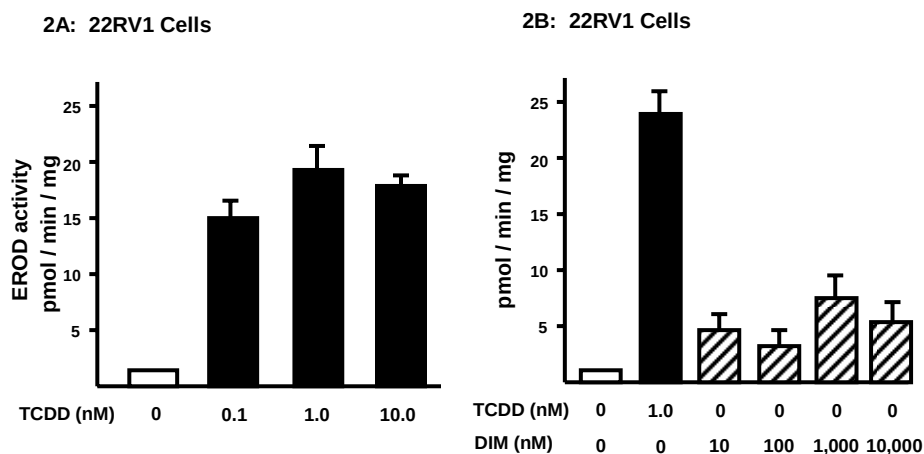


Figure 2. Induction of CYP1A1-dependent EROD activity by DIM and TCDD in 22RV1 prostate cancer cells.

The induction of EROD activity by TCDD and DIM in PC3 prostate cancer cells was also investigated using serum-free medium. The results after treatment for 24 hr showed that 10 nM TCDD and both 1 and 10 μ M DIM induced EROD activity (< 3 -fold) (Fig. 3). In contrast, 1 nM TCDD was not active. The experiments were therefore extended for 48, 72 and 96 hr, and the results showed the novel effects of prolonged treatment with TCDD or DIM on induction of EROD activity. The lower concentrations of TCDD (1 nM) and DIM (1 μ M) were not inducers at the longer time periods, and the induction response by 10 μ M DIM was similar at all time points. In contrast, prolonged treatment with 10 nM TCDD increased induction of EROD activity and after 96 hr, there was a > 29 -fold induction response. These results demonstrate that the AhR is functional in PC3 prostate cancer cells; however, optimal responsiveness is observed only after 96 hr and the reason for this unusual temporal pattern of Ah-responsiveness is currently being investigated.

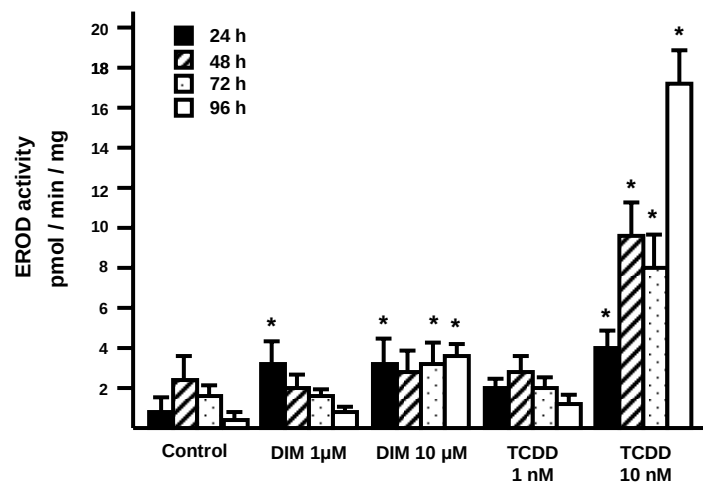
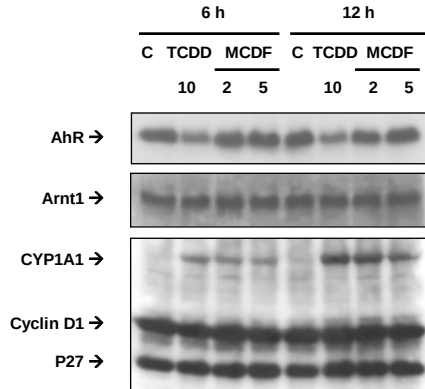
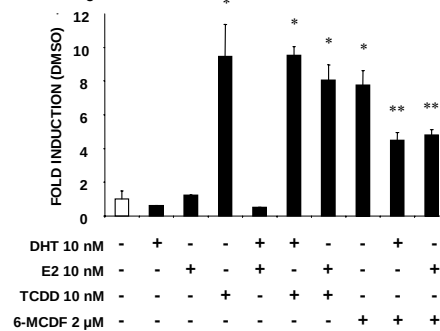


Figure 3. Induction of EROD activity by DIM and TCDD in PC3 prostate cancer cells. Cells were seeded 50,000 per well in 48-well plates in DME + 5% stripped serum and allowed to attach 24 h. Cells were then treated with DMSO vehicle or treatments in DMSO one time in DME-F12 media without serum supplementation, with 7 or 8 wells per treatment group. On separate plates, the EROD assay was performed after 24, 48, 72 or 96 h.

Western blot analysis also confirmed expression of both AhR and Arnt proteins, and treatment with TCDD but not 6-MCDF decreased expression of the AhR (Fig. 4A). Expression of other proteins including Sp1, cyclin D1 and p27 were unaffected by the treatments and serve as loading controls. Results illustrated in Figure 4B also show that treatment of LNCaP cells with 10 nM TCDD induced luciferase activity > 9-fold compared to solvent control (DMSO) in cells transfected with pDRE₃. In contrast, 10 nM DHT, 10 nM E2 and E2 plus DHT did not significantly induce activity, and neither DHT or E2 in combination with TCDD affected induced activity. 6-MCDF (2 μM), a prototypical SAhRM, also induced luciferase activity (> 7-fold), and this was consistent with the induction of CYP1A1 by 6-MCDF. 6-MCDF is a much less potent agonist for activation of CYP1A1 or DRE-dependent activities in breast cancer cells. Both E2 and DHT in combination with 6-MCDF significantly inhibited 6-MCDF-induced activity, whereas in cells treated with TCDD in combination with E2 or DHT, inhibitory interactions were not observed.

A. LNCaP cells

B. pDRE₃

C.

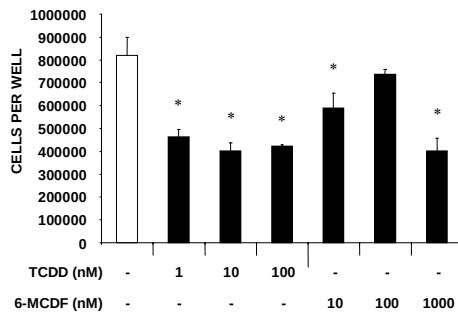


Figure 4. Ligand-dependent AhR activation and growth inhibition in LNCaP cells. [A] Induction of CYP1A1 protein. LNCaP cells were treated with DMSO (C), 10 nM TCDD, 2 or 5 μM 6-MCDF for 6 or 12 h, and whole cell lysates were analyzed by Western blot analysis as described in the Materials and Methods. Antibodies were used to detect the AhR, Arnt, CYP1A1, cyclin D1 and p27 proteins. [B] Activation of pDRE₃. LNCaP cells were transfected with pDRE₃, treated with various compounds and luciferase activity was determined as described in the Materials and Methods. Significant induction ($p < 0.05$) is indicated with an asterisk and inhibition of TCDD- or 6-MCDF-induced activity is also indicated (**). [C] Inhibition of LNCaP cell growth by TCDD and 6-MCDF. Cells were cultured for six days, treated with different concentrations of TCDD or 6-MCDF, and cell numbers were determined as described in the Materials and Methods. Significant ($p < 0.05$) growth inhibition is indicated by an asterisk. All results are presented as means $\pm$ SE for three replicate determinations for each treatment group. Growth inhibition in some of the groups was observed after 2 to 4 days.

The comparative effects of TCDD and 6-MCDF on growth of LNCaP cells were also determined in cells treated with solvent control and different concentrations of the AhR agonists for 6 days. The results show that TCDD (1 – 100 nM) significantly inhibited proliferation of LNCaP cells, and growth inhibition was also observed for 6-MCDF (Fig. 4B). Both compounds inhibited $\geq 50\%$ cell growth at one or more concentrations. Similar experiments were also carried out with 6-MCDF and TCDD in LNCaP cells also treated with different concentrations of DHT (up to 10 nM). Hormone-induced cell growth was not observed; however, both 6-MCDF and TCDD inhibited growth of LNCaP cells in the presence of DHT (data not shown). These

results confirm that LNCaP cells are Ah-responsive and both TCDD and 6-MCDF inhibit LNCaP cell proliferation. The effects of TCDD on cell cycle progression was also determined in LNCaP cells treated with 1.0, 10 and 100 nM TCDD for 48 hr followed by FACS analysis. The results show that TCDD induced a small but significant increase in the percentage of cells in G₀/G₁ and a decrease of cells in S phase, whereas minimal differences in distribution of cells in G₀/G₁, S and G₂/M phases were observed in LNCaP cells treated with solvent (DMSO) or DHT (10 nM). The results also show that DIM inhibits growth of PC3 cells in serum-free or 1% serum-containing medium at concentrations as low as 0.1 μ M DIM (Fig. 5). Similar results were obtained for both DIM and 6-methyl-1,3,8-trichlorodibenzofuran (6-MCDF) in 22RV1 cells (Fig. 6) and demonstrate that both classes of SAhRMs inhibit growth of prostate cancer cells.

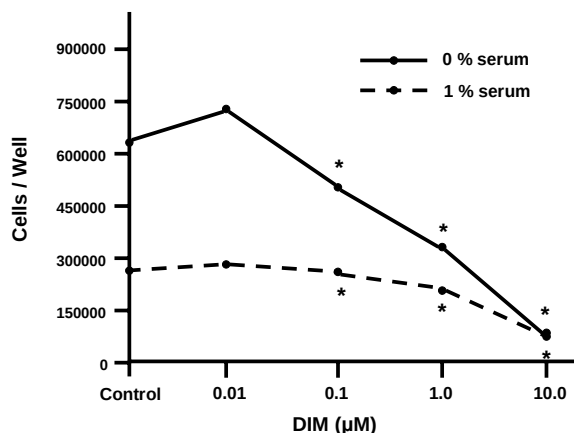


Figure 5. Inhibition of PC3 prostate cancer cell growth by DIM. Cells (10^5) were seeded in 6-well plates and treated with different concentrations of DIM for 6 days. Significant ($p < 0.05$) inhibition of cell growth is indicated by an asterisk and results are presented at means $\pm$ SE for at least three replicate determinations.

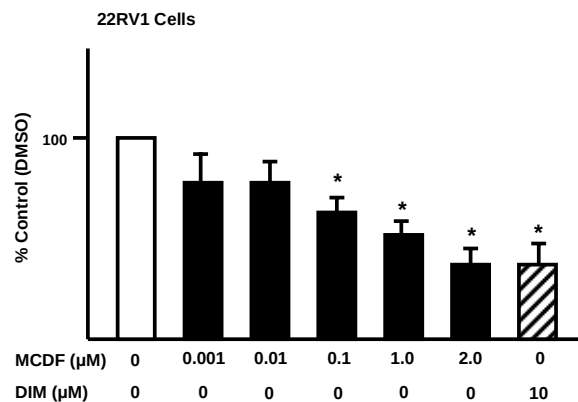


Figure 6. Inhibition of 22RV1 cell growth by 6-MCDF and DIM. Cells were essentially treated as described for PC3 cells (Fig. 4) and significant ($p < 0.05$) induction is indicated by an asterisk.

Inhibitory AhR-AR Crosstalk in LNCaP Cells Transfected with Androgen-responsive

Constructs

Effects of TCDD, dihydrotestosterone (DHT) and E2 on AR levels

The AR is expressed in 22RV1 prostate cancer cells, and we have investigated the time-dependent effects of DHT, 17 β -estradiol (E2), TCDD, E2 plus TCDD, and DHT plus TCDD on AR protein expression in this cell line (Task 2). Ligands for several receptors initiate degradation of their cognate receptors (37-46); for example, estrogens, retinoids and progestins trigger proteasome-dependent ER, RXR/RAR and PR protein downregulation. One report showed that androgens do not induce downregulation of AR protein in LNCaP cells (47); however, in untreated cells, the proteasome inhibitor MG132 enhanced AR protein levels in the same cell line. TCDD and SAhRMs induce proteasome-dependent degradation of the AhR and ER α in breast cancer cells, and we therefore investigated inhibitory AhR-AR crosstalk on AR protein expression. The results in Figure 7 demonstrate that 10 nM DHT induces a time-dependent > 3.5-fold increase in AR protein expression over a treatment period of 24 hr, whereas 10 nM E2 or TCDD alone (or in combination) had no affect on AR protein levels. In 22RV1 cells cotreated with TCDD plus DHT, the increased expression observed with DHT alone was repressed by TCDD, and similar results were observed in LNCaP cells (data not shown).

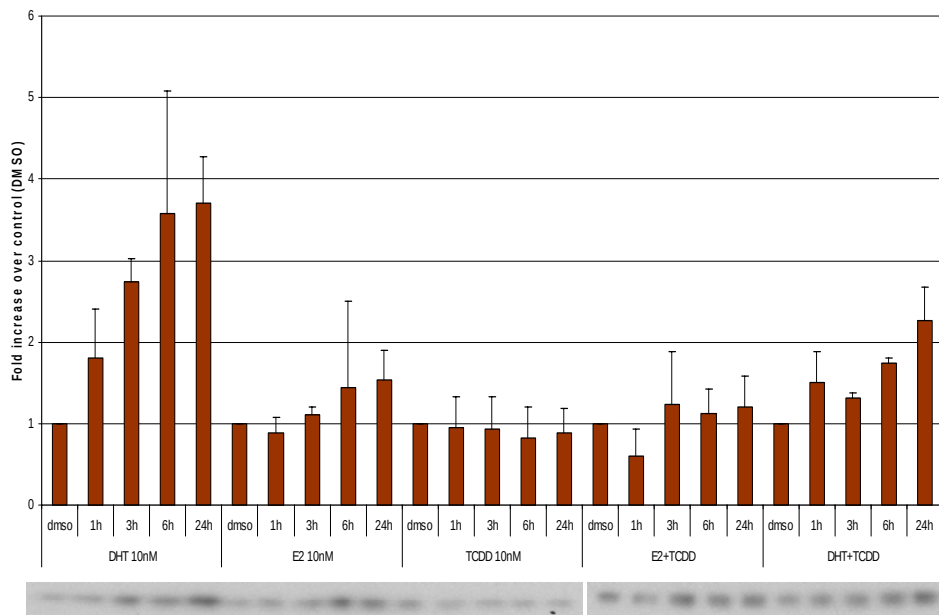


Figure 7. Effects of DHT, E2, TCDD and their combination on AR protein expression on 22RV1 cells. Results are expressed as means $\pm$ SD for at least three replicate determinations.

Jana and coworkers (27) previously reported that TCDD inhibited testosterone-induced luciferase activity in LNCaP cells transfected with an androgen-responsive construct containing the mouse mammary tumor virus (MMTV) promoter. Inhibition of testosterone-induced PSA protein or mRNA by 100 nM TCDD was reported but not quantitated, and the magnitude of inhibition was minimal. Therefore, we further investigated inhibitory AhR-AR crosstalk in LNCaP cells transfected with pPB which contains the -286 to +28 region of the androgen-responsive probasin gene promoter (Fig. 8A). There was a > 13 -fold increase in luciferase activity in LNCaP cells treated with 10 nM DHT and transfected with pPB and the induced response was significantly inhibited after cotreatment with DHT plus TCDD. Similar inhibitory responses were also observed using 2 μ M MCDF (Fig. 8A), whereas TCDD and MCDF alone did not significantly induce activity. Surprisingly, 10 nM E2 alone induces luciferase activity in LNCaP cells transfected with pPB, and the hormone-induced response is significantly decreased

in cells cotreated with E2 plus TCDD or 6-MCDF (Fig. 8A).

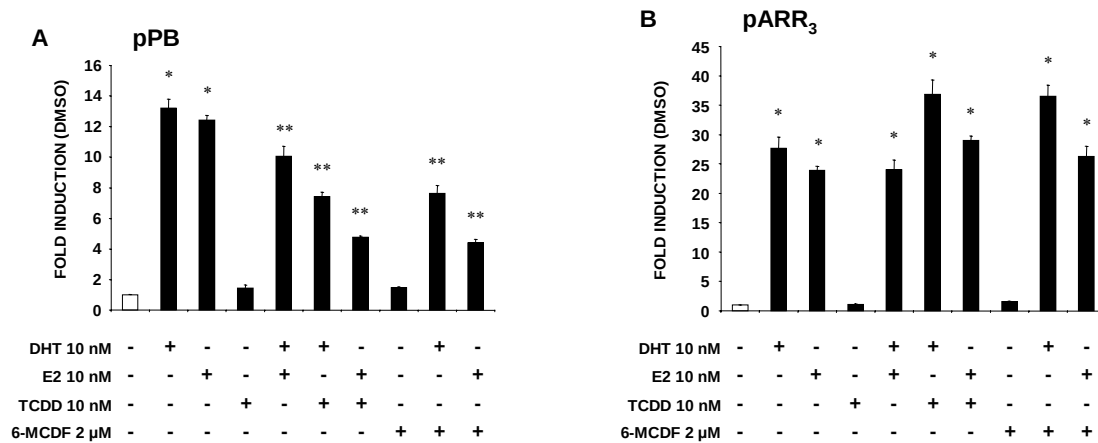


Figure 8. Inhibition of AR-dependent transactivation by TCDD and 6-MCDF. LNCaP cells were transfected with pPB [A] or pARR₃ [B], treated with hormone or AhR agonist alone or in combination, and luciferase activity was determined as outlined in the Materials and Methods. Significant ($p < 0.05$) induction by compounds alone is indicated by an asterisk, and significant ($0 < 0.05$) inhibitory effects observed in cotreatment studies are also indicated (**). Results are expressed as means $\pm$ SE for three replicate determinations for each treatment group.

The pARR₃ construct contains three tandem (3) copies of the probasin androgen response element, and was used to further investigate inhibitory AhR-AR crosstalk and the androgenic activity of E2. Ten nM DHT induced a > 27 -fold increase in luciferase in LNCaP cells transfected with pARR₃; however, for this construct, cotreatment with DHT plus MCDF or TCDD did not decrease DHT-induced activity (Fig. 2B). E2 (10 nM) also induced luciferase activity (> 24 -fold) in cells transfected with pARR₃; however, in cells cotreated with E2 plus TCDD or MCDF, activity was not significantly decreased compared to that observed for E2 alone. These results confirmed that both DHT and E2 activated gene expression in cells transfected pPB or pARR₃; however, inhibitory effects of AhR agonists were observed only for the former construct.

The unexpectedly high AR agonist activity of E2 compared to DHT in LNCaP cells were further investigated in cells transfected with pPB and treated with hormones and antiandrogens or antiestrogens. Induction of luciferase activity by 10 nM DHT and E2 in LNCaP cells

transfected with pPB was inhibited in cells cotreated with the hormone plus 10 μ M HPTE, an AR antagonist (Fig. 9A). However, in parallel studies, the "pure" antiestrogen ICI 182780 also significantly inhibited E2-induced activity, whereas only minimal inhibition was observed in LNCaP cells treated with DHT plus ICI 182780. In a parallel experiment in LNCaP cells transfected with pARR₃, both HPTE and ICI 182780 inhibited DHT and E2-induced luciferase activity (Fig. 9B), whereas 1 μ M flutamide, an AR antagonist, caused only minimal decreases in hormone-induced activity (Fig. 9C). HPTE is also an ER α agonist and ER β antagonist, and the results obtained for both HPTE and ICI 182780 suggest a possible role for ER β in mediating activation of pPB and pARR₃. However, previous studies show that endogenous ER β is insufficient for E2-induced transactivation in LNCaP cells transfected with pERE₃, a construct containing three tandem estrogen responsive elements (ERE₃), suggesting that activation of pPB or pARR₃ is ER β -independent. Therefore, in order to confirm the role of AR in mediating these responses, we further investigated hormone activation of pPB and inhibitory AhR-AR crosstalk in ZR-75 cells which express minimal AR protein.

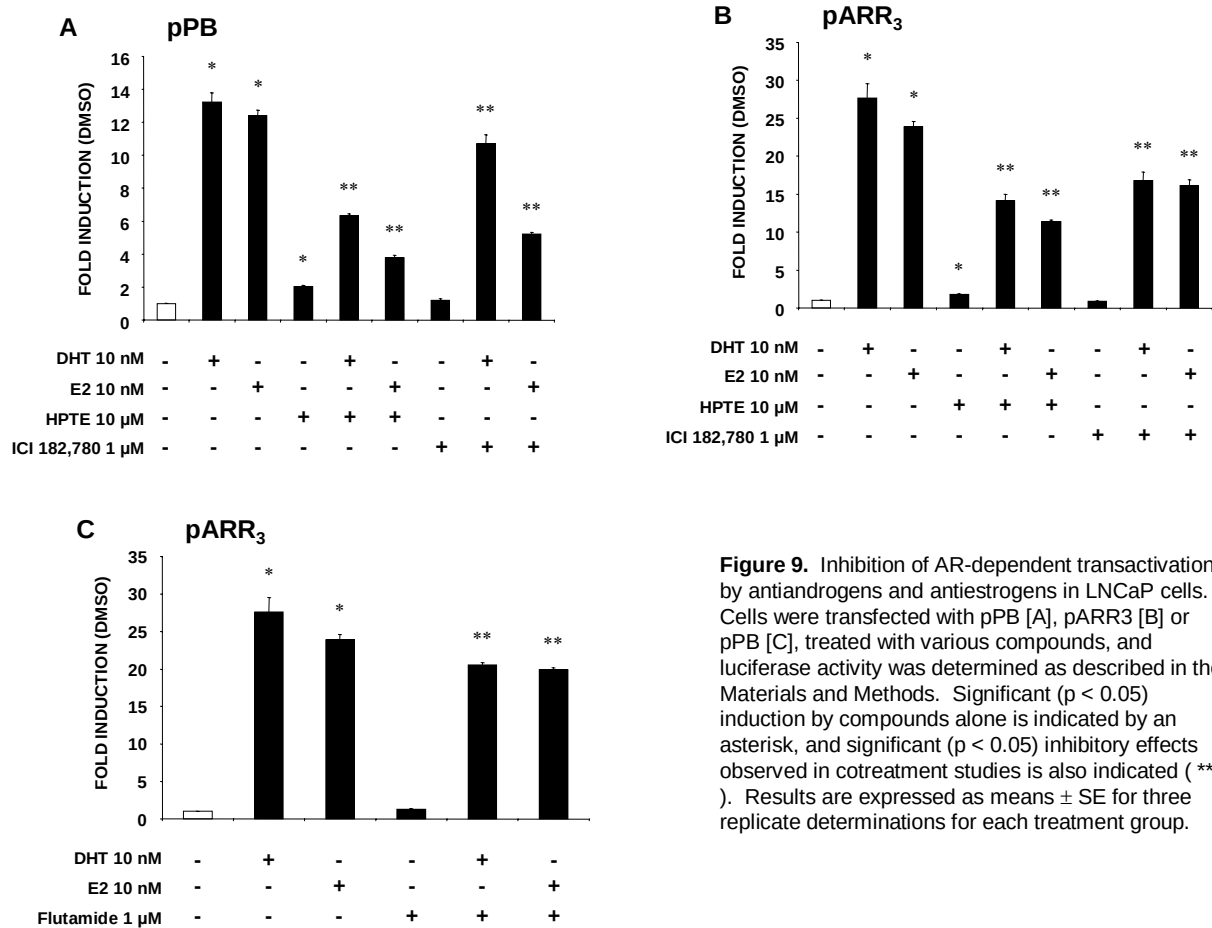


Figure 9. Inhibition of AR-dependent transactivation by antiandrogens and antiestrogens in LNCaP cells. Cells were transfected with pPB [A], pARR3 [B] or pPB [C], treated with various compounds, and luciferase activity was determined as described in the Materials and Methods. Significant ($p < 0.05$) induction by compounds alone is indicated by an asterisk, and significant ($p < 0.05$) inhibitory effects observed in cotreatment studies is also indicated (**). Results are expressed as means $\pm$ SE for three replicate determinations for each treatment group.

Results in Figure 10A show that DHT, E2, TCDD and MCDF do not activate reporter gene activity in ZR-75 cells transfected with pPB alone; however, both DHT and E2 induced luciferase activity in cells cotransfected with pPB and hAR expression plasmid (Fig. 10B). Induction by E2 was significant but lower than observed for DHT in ZR-75 cells, and TCDD inhibited E2 but not DHT-induced activity in cells cotreated with hormone plus TCDD. Similar results were observed in duplicate experiments confirming that E2-dependent transactivation of pPB was AR-dependent. However, it was also evident that there were important differences between the interaction of TCDD and DHT in LNCaP and ZR-75 cells since TCDD did not inhibit DHT-induced luciferase activity in the latter cell line. This suggests that inhibitory AhR-AR crosstalk is cell context-dependent for the pPB promoter.

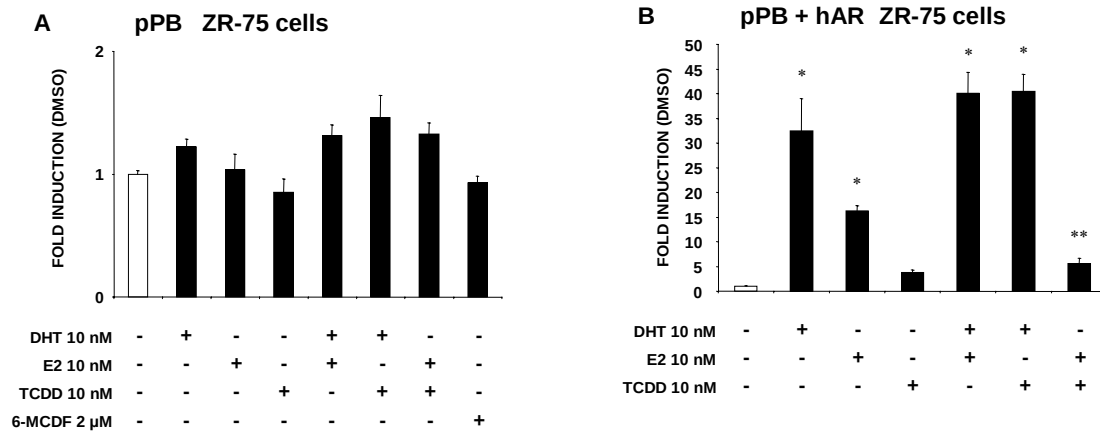


Figure 10. Inhibition of hormone-induced transactivation in ZR-75 breast cancer cells transfected with pPB. [A] Transfection with pPB alone. ZR-75 cells were transfected with pPB, treated with various compounds and luciferase activity was determined as described in the Materials and Methods. No significant induction was observed in any of the treatment groups. [B] Transfection with pPB and hAR. Cells were transfected and treated as described in [A] except that 500 ng of hAR expression plasmid was also transfected. Significant ($p < 0.05$) induction by compounds alone is indicated by an asterisk and significant inhibitory effects observed in cotreatment studies is also indicated (**). Results are expressed as means $\pm$ SE for three replicate determinations for each treatment group.

Effects of Various Treatments on AR, Cyclin D1 and p27 Protein Levels in LNCaP Cells

Levels of AR protein expression may influence androgen-responsiveness and inhibitory AhR-AR crosstalk, and the results in Figure 5A demonstrate levels of immunoreactive AR protein in LNCaP cells after various treatments. Preliminary studies in LNCaP and other cell lines indicated that any changes in AR expression were observed within 6 - 12 hr after treatment (data not shown) and a 6 hr time point was selected for this study. Treatment with 10 nM DHT, 10 nM E2 or DHT plus E2 resulted in a significant increase in AR levels. In contrast, 10 nM TCDD and 2 μ M 6-MCDF alone did not significantly affect levels of AR protein; however, in combination with DHT, there was a significant decrease in AR levels compared to cells treated with DHT alone. TCDD in combination with E2 also decreased AR levels compared to those observed in cells treated with E2 alone. In contrast, levels of immunoreactive p27 protein were not significantly changed by any of the treatments, and served as a loading control for this

experiment. In a separate study, the effects of the antiandrogen HPTE and the antiestrogen ICI 182780 alone and in combination with E2 or DHT on AR levels were also determined (Fig. 11B). Ten μ M HPTE alone did not affect AR levels in LNCaP cells, whereas ICI 182780 treatment increased AR levels compared to DMSO (solvent) treatment. Hormone (E2 or DHT)-induced upregulation of AR protein was not decreased cotreatment with HPTE or ICI 182780. Cyclin D1 protein was not significantly changed in this study and served as a loading control. These data demonstrate that various treatments differentially modulate AR protein levels in LNCaP cells, and current studies are focused on the influence of ligand-induced changes in AR expression and the magnitude of hormone-induced transactivation.

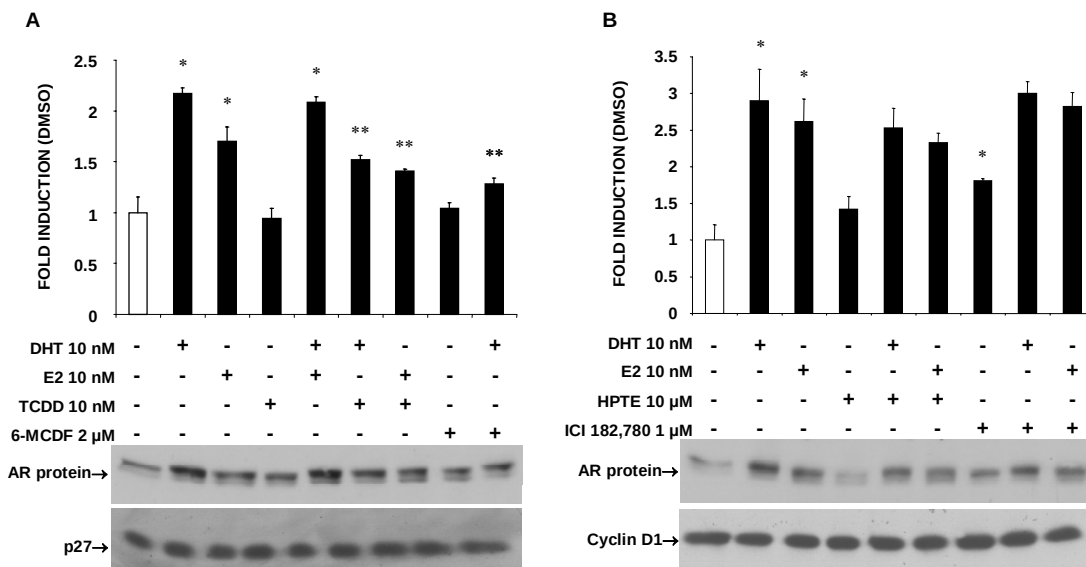


Figure 11. AR protein expression in LNCaP cells treated with hormones, AhR agonists, antiandrogens and antiestrogens. [A] AR protein expression in cells treated with hormones and AhR agonists. LNCaP cells were treated with DHT, E2, TCDD, 6-MCDF and their combinations for 6 h, and AR protein levels in whole cell lysates were determined by Western blot analysis as described in the Materials and Methods. p27 protein was also determined for this experiment; p27 was essentially unchanged in all of the treatment groups and serves as a loading control for this experiment. [B] AR protein expression in cells treated with hormones, antiandrogens and antiestrogens. AR protein levels were determined essentially as described in [A] and blots were stripped and reprobed with cyclin D1 antibodies. Cyclin D1 protein was unchanged in this experiment and serves as a loading control. For studies illustrated in [A] and [B], significant ($p < 0.05$) increases in AR protein levels by individual compounds are indicated by an asterisk, and significant ($p < 0.05$) decreases in the cotreatment groups are also indicated (**). Results are expressed as means $\pm$ SE for three replicate determinations for each treatment group.

Dim and Ring-Substituted DIMs: AR Agonist and Antagonist Activities in Transactivation

Assays

Previous studies show that DIM exhibits AR antagonist activity in LNCaP cells and inhibits DHT-induced PSA protein and reporter gene activity in cells transfected with androgen-responsive constructs (48). In this study, the antiandrogenic activity of DIM has been investigated in LNCaP and 22Rv1 prostate cancer cells transfected with the androgen-responsive pPB construct (Fig. 12). Both cell lines express mutant forms of the AR; however, DHT activates the receptor in LNCaP and 22Rv1 cells (49, 50). The results show that DHT but not DIM alone significantly induced luciferase activity in LNCaP and 22Rv1 cells transfected with pPB. In cells cotreated with DHT plus DIM and transfected with pPB, hormone-induced luciferase activity was significantly decreased at DIM concentrations of 5, 10 and 20 μ M. These results confirm that DIM inhibits activation of the androgen responsive pPB construct by DHT in both LNCaP and 22Rv1 cells and complements results of a previous report on the antiandrogenic activity of DIM (12).

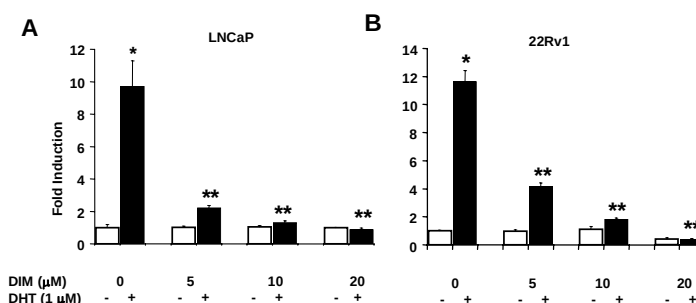


Figure 12. Antiandrogenic activity of DIM. LNCaP (A) or 22Rv1 (B) cells were transfected with pPB, treated with DHT, 5 - 20 μ M DIM alone or in combination with DHT, and luciferase activity determined as described in the Materials and Methods. Results are expressed as means $\pm$ SE for at least 3 determinations per treatment group, and significant ($p < 0.05$) induction (*) or inhibition (**) in the cotreatment groups are indicated.

Ring-substituted DIMs also exhibit potent anticancer activities (33, 35); however, the structure-dependent effects of these compounds as antiandrogens have not been reported. This study investigates the AR antagonist and agonist activities of symmetrical dihaloDIMs

containing substituents in the 4, 5, 6, and 7 positions of the benzene ring. The structure-dependent AR antagonist/agonist activities of 4,4'-, 5,5'-, 6,6'-, and 7,7'-dichloro- and -dibromoDIMs were investigated in LNCaP and 22Rv1 cells transfected with the PB construct (Figs. 13 and 14). The dichloro- and dibromoDIM isomers induce similar structure-dependent responses in both cell lines. The results obtained for the dichloroDIM isomers show that 4,4'-, 5,5'-, and 6,6'-dichloroDIM were AR antagonists in LNCaP and 22Rv1 cells (Figs. 13A and 13B), although their AR antagonist activity was more pronounced in the latter cell line. 7,7'-DichloroDIM was a partial AR agonist/antagonist in both cell lines (Figs. 13C and 13D), whereas the other isomers did not exhibit AR agonist activities. The pattern of

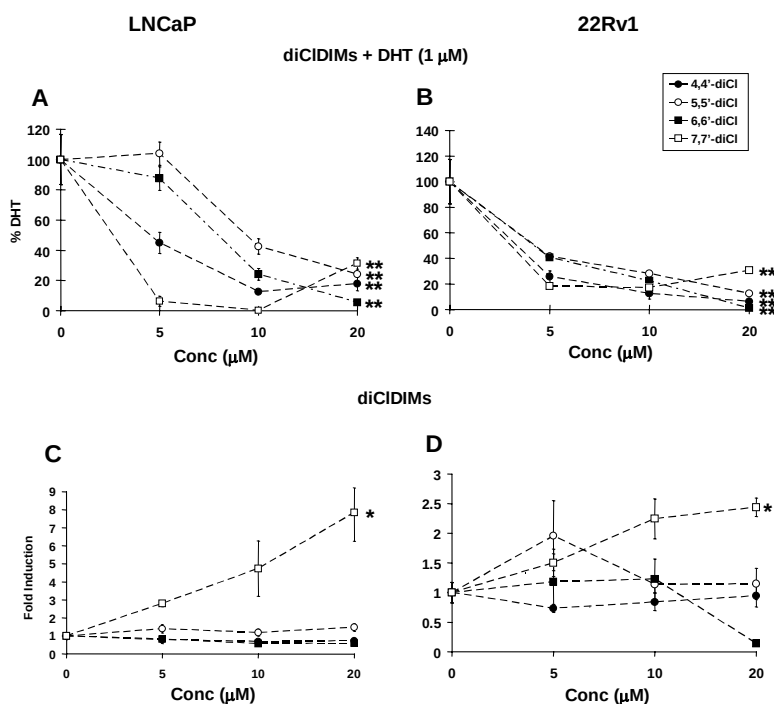


Figure 13. Antiandrogenic and androgenic activity of isomeric dichloroDIMs in LNCaP (A, C) and 22Rv1 (B, D) cells. Cells were transfected with pPB, treated with DHT, 5 to 20 μ M dichloroDIMs alone or in combination with DHT, and luciferase activity determined as described in the Materials and Methods. Results are expressed as means $\pm$ SE for at least 3 determinations per treatment group, and significant ($p < 0.05$) induction (*) or inhibition (**) in the 20 μ M cotreatment groups are indicated.

antiandrogenic/androgenic activities for the isomeric dichloroDIMs (Fig. 14) was similar to that observed for the brominated analogs (Fig. 3). 4,4'-, 5,5'- and 6,6'-DibromoDIM primarily exhibited antiandrogenic activities in LNCaP and 22Rv1 cells (Figs. 14A and 14B), and 7,7'-dibromoDIM was a partial AR agonist/antagonist in both cell lines (Figs. 14C and 14D). In addition, all of the dihaloDIMs inhibit LNCaP cell proliferation with inhibitory IC_{50} values of 1 - 5 μ M (data not shown).

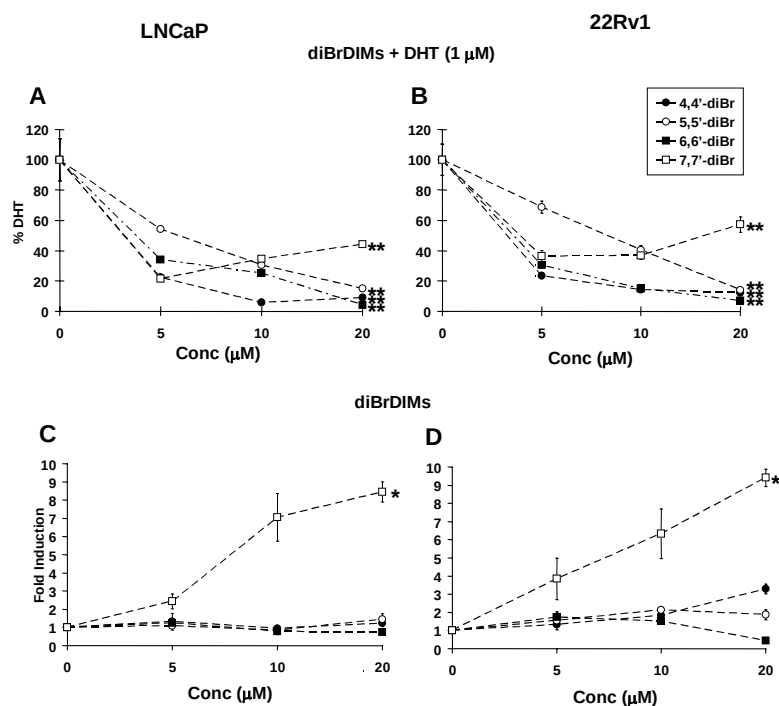


Figure 14. Antiandrogenic and androgenic activity of isomeric dibromoDIMs in LNCaP (A, C) and 22Rv1 (B, D) cells. Cells were trans-fected with pPB, treated with DHT, 5 - 20 μ M dibromoDIMs alone or in combination with DHT, and luciferase activity determined as described in the Materials and Methods. Results are expressed as means $\pm$ SE for at least three determinations per treatment group, and significant ($p < 0.05$) induction (*) or inhibition (**) in the 20 μ M cotreatment groups are indicated.

Structure-dependent Effects of Isomeric DihaloDIMs on AR Protein Expression

The antiandrogenic activity of DIM was associated with inhibition of DHT-induced formation of nuclear AR ; however, other compounds such as tea polyphenols and emodin inhibit androgen responsiveness through downregulation of AR protein (51, 52). We therefore

investigated the effects of the dichloroDIM (Fig. 15A) and dibromoDIM (Fig. 15B) isomers on AR protein expression in LNCaP cells. Cells were treated with different concentrations of the individual compounds for 24 hr and whole cell lysates were analyzed for AR protein by Western blot analysis. 4,4'- and 5,5'-DichloroDIM (up to 20 μ M) did not affect AR protein levels; however, AR protein expression was decreased by both 6,6'- and 7,7'-dichloroDIM. Results are shown only for 15 μ M 6,6'-dichloroDIM due to the high cytotoxicity of this compound. Results in Figure 15B for the dibromoDIMs gave a similar pattern of isomer-dependent responses, namely 4,4'-dibromoDIM had minimal effects on levels of AR protein, whereas both 6,6'- and 7,7'-dibromoDIM decreased AR levels. 5,5'-DibromoDIM also decreased expression of AR protein but only at the 20 μ M concentration. These results demonstrate remarkable differences in the effects of 4,4'-/5,5'-dihaloDIMs and 6,6'-/7,7'-dihaloDIMs on AR protein expression in LNCaP cells, and these differences were further investigated using the 4,4' and 7,7'-dihaloDIM as prototypes.

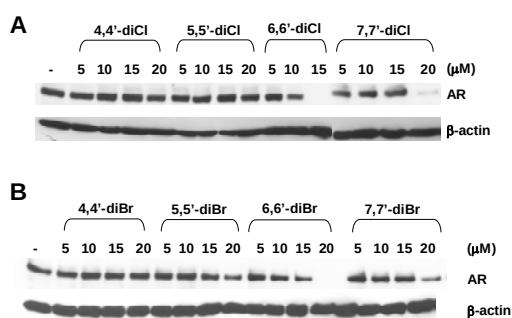


Figure 15. Structure-dependent effects of isomeric dihaloDIMs on AR protein levels. LNCaP cells were treated with different concentrations of isomeric dichloroDIMs (A) and dibromoDIMs (B) for 24 hr, and whole cell lysates were analyzed for AR and β -actin (loading control) protein by Western blot analysis as described in the Materials and Methods.

Previous studies showed that nuclear levels of AR increased after treatment of LNCaP cells with DHT for 24 hr, and DHT-induced nuclear translocation of AR was inhibited after cotreatment with DIM (48). Figure 16A summarizes the effects of 20 μ M 4,4'- and 7,7'-dichloroDIM, 20 μ M DIM and 10 nM DHT on cytosolic and nuclear AR levels after treatment for 1 and 24 hr. Minimal changes in cytosolic (c) and nuclear (n) AR levels were observed in all treatment groups (compared to DMSO) after 1 hr, and no major changes in AR protein staining in the cytosolic or nuclear fractions were observed. In cells treated for 24 hr, DHT induced a more intense staining of AR in the nuclear fraction and enhanced overall AR staining (c+n) compared to cells treated with DMSO alone (c+n). This was observed in replicate experiments and is consistent with results of previous studies showing that DHT enhanced AR expression in LNCaP cells (53, 54). Both DIM and 4,4'-dichloroDIM alone also enhanced AR levels in both the cytosolic and nuclear fractions; however, in combination with DHT, these compounds did not block DHT-induced formation of nuclear AR, and this was in contrast to a previous report showing that DIM inhibited this response (48). In contrast, 7,7'-dichloroDIM alone decreased nuclear and cytosolic AR levels after treatment for 24 hr and, in combination (DHT + 7,7'-dichloroDIM), the DHT-induced nuclear AR levels were only slightly decreased. The observed downregulation of AR protein in both the cytosolic and nuclear fractions (Fig. 16A) complements the results observed for AR levels in whole cell lysates from cells treated with 7,7'-dichloroDIM (Fig. 16A). Sp1 protein served as a loading control for this study, and the identification of Sp1 only in the nuclear fraction confirms the efficiency of the separation of nuclear and cytosolic fractions.

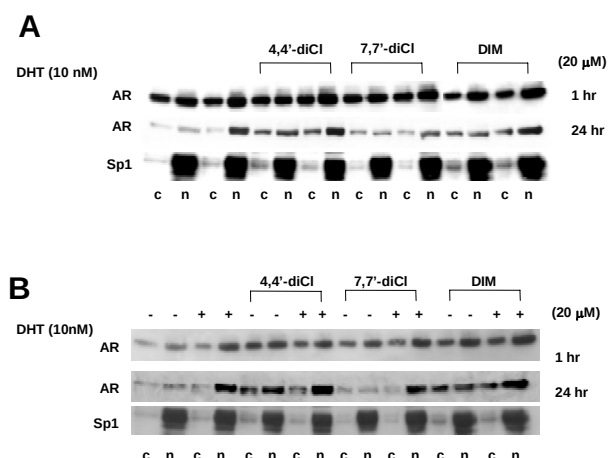


Figure 16. Cytosolic (c) and nuclear (n) AR protein in LNCaP cells treated with DIM and dihaloDIMs. (A) Treatment with DIM, 4,4'- and 7,7'-dichloro-DIM. Cells were treated with DMSO, DHT, 4,4'- and 7,7'-dichloroDIM or DIM alone or in combination with DHT for 1 or 24 hr, and cytosolic or nuclear fractions were obtained and analyzed by Western blot analysis as described in the Materials and Methods. The nuclear Sp1 protein was determined as a loading control and to determine the efficiency of the isolation of the cytosolic and nuclear fractions. (B) Treatment with DIM, 4,4'- and 7,7'-dibromoDIM. Cells treated with DMSO, DHT, 4,4' or 7,7'-dibromoDIM alone or in combination with DHT for 48 hr, and nuclear and cytosolic fractions were analyzed by Western blot analysis as described in the Materials and Methods. Nuclear Sp1 protein serves as a control for determining the efficiency of the isolated cytosolic and nuclear fractions.

In two separate experiments (1 and 24 hr), the effects of DMSO, DHT, 4,4'- and 7,7'-dibromoDIM on AR levels were determined in LNCaP cells (Fig. 16B). The pattern of effects for the dibromoDIMs alone, and in combination with DHT was similar to those observed for the dichloroDIM isomers. DHT and 4,4'-dibromoDIM induced a time-dependent increase in AR levels (compared to DMSO). Interaction of 4,4'-dibromoDIM with DHT decreased the ratio of nuclear/cytosolic levels of AR; however, this could be an additive effect since the former compounds alone induced higher cytosolic AR levels. In contrast, cytosolic and nuclear AR protein levels were decreased after treatment with 7,7'-dibromoDIM alone for 24 hr. In cells cotreated with 7,7'-dibromoDIM plus DHT, AR levels and their distribution were similar to those observed for DHT alone. Both 7,7'-dibromo- and 7,7'-dichloroDIM appeared to induce a time-dependent decrease in AR protein, whereas DHT, DIM and 4,4'-dichloro-, and 4,4'-dibromoDIM increased or stabilized AR protein in LNCaP cells. This was further investigated in LNCaP cells treated for 48 hr with DHT, 4,4'- and 7,7'-dihaloDIMs followed by Western blot analysis of whole cell lysates. Results in Figure 17A confirm that 7,7'-dichloro- and 7,7'-dibromoDIM decreased AR protein expression (compared to DHT), whereas AR levels after treatment with

the corresponding 4,4'-dihaloDIMs (Fig. 17B) were significantly higher than observed in cells treated with the 7,7'-dihaloDIMs (Fig. 17A). We also observed that Sp1 protein was also slightly decreased only after prolonged treatment with the 7,7'-dihaloDIMs.

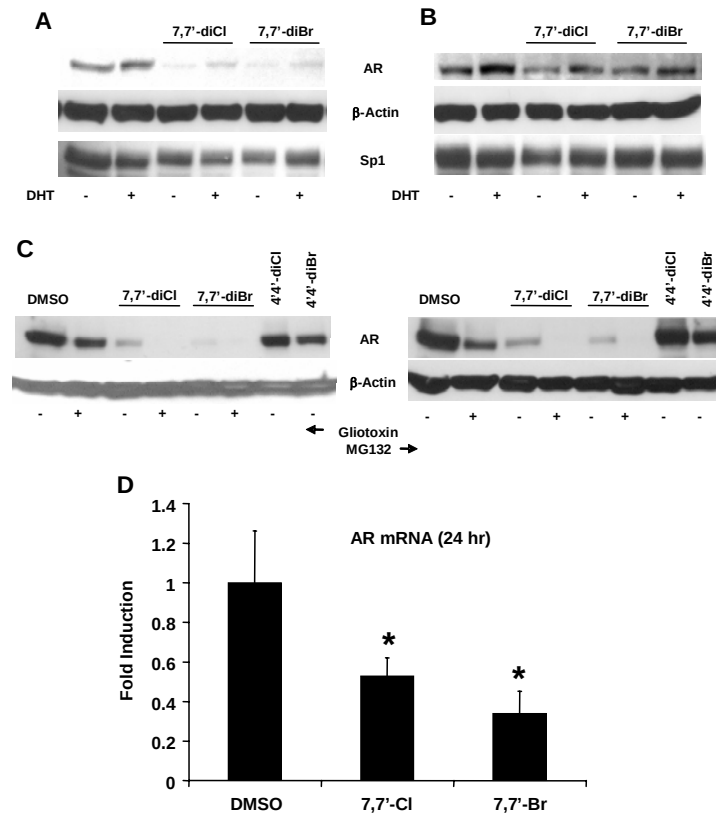


Figure 17. Effects of 4,4'- or 7,7'-dihaloDIM on AR expression and androgen responsiveness. Effects of 7,7'-dihaloDIMs (A) and 4,4'-dihaloDIMs (B) on AR protein levels. LNCaP cells were treated with DMSO, 10 nM DHT, 20 μ M dihaloDIMs for 48 hr, and whole cell lysates were analyzed by Western blot analysis as described in Materials and Methods. AR, β -actin (loading control) and Sp1 protein were determined. (C) Effects of proteasome inhibitors on AR protein levels. LNCaP cells were treated with 20 μ M 7,7'-dichloro- or 7,7'-dibromo DIM alone or in combination with the proteasome inhibitor gliotoxin (3 μ M) or MG132 (10 μ M) for 48 hr, and AR protein levels were determined by Western blot analysis as described in Materials and Methods. β -Actin served as a loading control. (D) Time-dependent effects of 7,7'-dihaloDIMs on AR mRNA protein levels. LNCaP cells were treated with 7,7'-dihaloDIMs for 24 hr, and AR mRNA was determined by real-time PCR as described in the Materials and Methods. The experiments were carried out in triplicate. Results are expressed as means $\pm$ SE, and significantly ($p < 0.05$) decreased AR mRNA is indicated by an asterisk. TBP mRNA was also determined and used to normalize the AR mRNA levels.

Effects of DihaloDIM Isomers on AR Protein and mRNA Levels and Their Antiandrogenic Activities in LNCaP Cells

The potential role of proteasome activation in mediating downregulation of AR was investigated. LNCaP cells were treated with 7,7'-dihaloDIMs for 48 hr in the presence or absence of the proteasome inhibitors gliotoxin or MG132 (Fig. 17C). Western blot analysis of whole cell lysates showed that 7,7'-dichloro- and 7,7'-dibromoDIM significantly decreased AR protein compared to levels observed in solvent-treated cells, and cotreatment with the proteasome inhibitors further increased AR degradation. The proteasome inhibitors alone also decreased AR protein, whereas 4,4'-dichloro- and 4,4'-dibromoDIM did not affect AR protein. The data indicated that decreased AR protein in LNCaP cells treated with 7,7'-dihaloDIMs is not due to activation of the proteasome pathway. The time-dependent effects of 7,7'-dihaloDIMs on AR mRNA levels was determined (Fig. 17D), and the results show that mRNA levels are significantly decreased within 24 and 48 hr (data not shown). We also investigated the effects of 7,7'-dichloroDIM on AR mRNA stability by pretreating cells with DMSO or 20 μ M 7,7'-dichloroDIM for 12 hr prior addition of actinomycin D. The results showed an initial 6 - 12 hr increase in AR mRNA levels after addition of actinomycin D; however, the subsequent rates of degradation of AR mRNA in the DMSO and 7,7'-dichloroDIM treatment groups were comparable (data not shown). These data indicate that 7,7'-dihaloDIMs decrease both transcriptional and translational regulation of the AR.

Results of transient transfection studies showed that both 7,7'-dibromo- and 7,7'-dichloroDIM were partial AR agonists and AR antagonists (Figs. 13 and 14), and the former was observed after treatment for 36 hr. This AR agonist activity of 7,7'-dihaloDIMs is inconsistent with their effects on AR and it is possible that 7,7'-dihaloDIM-induced androgenic activity after

36 hr (Figs. 13 and 14) may be due to the relatively slow rate of AR degradation. We therefore investigated the time-dependent effects of 7,7'-dihaloDIMs on androgen-responsiveness in LNCaP cells transfected with pPB (for 9 hr), and then treated with different concentrations of 7,7'-dihaloDIMs for 24, 36 or 48 hr (Fig. 18A). The results indicated that after 36 hr, 5 - 20 μ M 7,7'-dichloroDIM significantly induced luciferase activity; however, this response was significantly decreased after treatment for 24 or 48 hr and similar results were observed for 7,7'-dibromoDIM (data not shown). The decreased AR agonist activity of 7,7'-dichloroDIM after 48 hr is consistent with the effects of this compound on AR protein degradation (Fig. 17A).

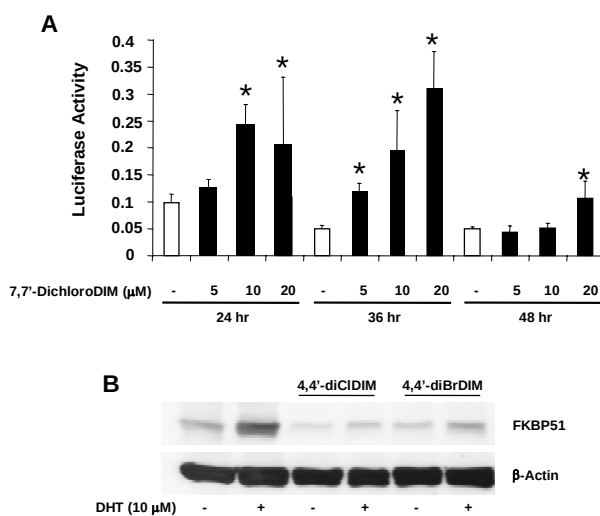


Figure 18. AR agonist/antagonist activities of dihaloDIMs. (A) Time-dependent effects of 7,7'-dichloroDIM on transactivation. LNCaP cells were transfected with pPB and, after 9 hr, were treated with 7,7'-dichloroDIM (5 - 20 μ M) for 24, 36 and 48 hr, and luciferase activity was determined as described in Materials and Methods. Results are expressed as means $\pm$ SE for at least 3 determinations for each treatment group and significant ($p < 0.05$) induction (*) is indicated. (B) Regulation of FKBP51 protein expression. LNCaP cells were treated with DMSO, 10 nM DHT, 4,4'-dichloro- or 4,4'-dibromoDIM alone or in combination with DHT for 48 hr, and whole cell lysates were analyzed by Western blot analysis for FKBP and β -actin (loading control) as described in Materials and Methods.

A recent study identified a 51 kDa progesterone receptor-associated immunophilin, FKBP51, as an androgen-responsive gene in prostate cancer cells (55), and the effects of 4,4'-dichloro- and 4,4'-dibromoDIM alone and in combination with DHT were investigated in LNCaP cells 48 hr after treatment (Fig. 18B). DHT alone enhanced FKBP51 protein expression, whereas 4,4'-dichloro- and 4,4'-dibromoDIM did not affect levels of FKBP51. In cells cotreated with DHT plus 4,4'-dichloro- or 4,4'-dibromoDIM, the hormone-induced response was inhibited by both DIM compounds and this was consistent with their antiandrogenic activity in transactivation assays (Figs. 13 and 14). Minimal induction of FKBP51 was observed after treatment with 7,7'-dichloro- or 7,7'-dibromoDIM (data not shown) and this may be due, in part, to the low levels of AR expression in LNCaP cells treatment with these compounds for 48 hr. In summary, these results indicate that ring-substituted DIMs and DIM differentially modulate androgenic responses in prostate cancer cells and subtle changes in the position of the ring substituents of the dihaloDIMs (i.e. 4 vs. 7) can modulate their mechanisms of antiandrogenic action and effects on AR expression.

KEY RESEARCH OUTCOMES

- 22RV1 prostate cancer cells have been identified as Ah-responsive.
- PC3 prostate cancer cells are also Ah-responsive but this is dependent on a lag time for activation of CYP1A1-dependent activity.
- SAhRMs inhibit growth of both 22RV1 and PC3 prostate cancer cells.
- Growth of LNCaP prostate cancer cells is inhibited by TCDD and 6-MCDF.
- DHT induces upregulation of AR protein in 22RV1 and LNCaP cells.
- E2 and TCDD do not affect AR protein expression in 22RV1 cells.

- TCDD partially blocks DHT-dependent upregulation of AR protein in 22RV1 cells.
- Inhibitory AhR–AR crosstalk was observed in LNCaP cells transfected with a construct (pPB) containing the androgen-responsive probasin promoter (-288 to +28).
- E2 was a potent androgen in LNCaP cells.
- E2 and DHT stabilized AR protein levels.
- AhR agonists partially inhibited stabilization of AR protein.
- Ring-substituted DIMs exhibit both antiandrogenic and androgenic activities in prostate cancer cells.
- These compounds also inhibit cell growth at concentrations ranging from 1 - 5 μ M (IC_{50} values) (data not shown).
- Among the dihaloDIMs, there was a structure-dependent effect on their degradation of the AR; 7,7'-dichloro- and 7,7'-dibromoDIM induced downregulation of AR protein, whereas the 4,4'-dihaloDIMs did not induce this response.
- The structure-dependent effects of the isomeric ring-substituted DIMs provides at least two mechanism-based classes of compounds that block prostate cancer cell growth through AR-dependent and - independent pathways.

REPORTABLE OUTCOMES

Safe, S. and McDougal, A. Mechanism of action and development of selective aryl hydrocarbon receptor modulators for treatment of hormone-dependent cancers. *Int. J. Oncol.* 20:1123-1128, 2002.

Morrow, D., McDougal, A. and Safe, S. Comparative aryl hydrocarbon receptor-hormone receptor crosstalk in breast and prostate cancer cells. Society of Toxicology Annual

Meeting, Nashville, TN. March, 2002. Abstract #LB31.

Morrow, D., Qin, C., Smith III, R. and Safe, S. Aryl hydrocarbon receptor-mediated inhibition of LNCaP prostate cancer cell growth and hormone-induced transactivation. *J. Steroid Biochem. Mol. Biol.* 88:27-36, 2004.

Morrow, D. and Safe, S. Aryl hydrocarbon receptor agonists inhibit hormone-induced transactivation in prostate cancer cells. Society of Toxicology Annual Meeting, Salt Lake City, UT, 2003. Abstract #1785.

Morrow, D. and Safe, S. Inhibitory aryl hydrocarbon receptor-androgen receptor crosstalk in 22Rv1 cells. Manuscript in preparation.

Kotha, L. and Safe, S. Structure-dependent androgen receptor agonist/antagonist activities and AR downregulation by dichloro- and dibromo-substituted 1,1-bis(3'-indolyl)methanes. *Mol. Carcinog.* submitted, 2006.

CONCLUSIONS

Initial studies have demonstrated that PC3, 22RV1 and LNCaP prostate cancer cells are Ah-responsive, and SAhRMs inhibit growth of these cells. TCDD also inhibits DHT-induced AR protein expression in 22RV1 cells. Similar results were obtained with the selective AhR modulator 6-methyl-1,3,8-trichlorodibenzofuran (6-MCDF); however, TCDD but not 6-MCDF induced degradation of the AhR protein. TCDD and 6-MCDF inhibited growth of LNCaP cells, and inhibitory AhR-androgen receptor (AR) crosstalk was investigated in cells transfected with constructs containing the androgen-responsive probasin promoter (-288 to +28) (pPB) or three copies of the -244 to -96 region of this promoter (pARR₃). Ten nM dihydrotestosterone (DHT) and 17 β -estradiol (E2) induced transactivation in LNCaP cells transfected with pPB or pARR₃;

however, inhibitory AhR-AR crosstalk was observed only with the latter construct. 6-MCDF and TCDD did not inhibit DHT- or E2-induced transactivation in ZR-75 human breast cancer cells, indicating that these interactions were promoter and cell context-dependent. Both E2 and DHT stabilized AR protein in LNCaP cells; however, cotreatment with TCDD or 6-MCDF decreased AR protein levels. These results indicate that inhibitory AhR-AR crosstalk in prostate cancer cells is complex and for some responses, AR protein stability may play a role.

Previous studies showed that DIM and ring-substituted DIMs are AhR agonists and DIM also exhibits antiandrogenic activity. Ongoing studies in the laboratory suggest that the growth inhibitory/proapoptotic effects of these compounds in breast cancer cells is AhR-independent. Results of this study do not exclude a role for the AhR in mediating responses; however, the unique and novel effects of ring-substituted DIMs in prostate cancer cells probably involves their direct effects on the AR and other subcellular targets. This study reports the structure-dependent androgenic/antiandrogenic activity of several symmetrical dichloro- and dibromoDIM isomers. Initial transactivation studies in LNCaP and 22Rv1 cells transfected with an androgen responsive construct (pPB) containing a probasin promoter insert showed that both 7,7'-dichloro- and 7,7'-dibromoDIMs exhibited partial androgenic activity. Most of the other isomeric substituted DIMs, including 4,4'-dichloroDIM and 4,4'-dibromoDIM, exhibited antiandrogenic activity in the transactivation assay. Structure-dependent differences were also observed for the effects of 4,4'- and 7,7'-dihaloDIMs on AR expression in LNCaP cells. Like DIM, 4,4'-dichloroDIM and 4,4'-dibromoDIM did not affect AR protein levels for up to 48 hr and inhibited dihydrotestosterone (DHT)-induced responses without affecting cytosolic or nuclear AR distribution. In contrast, the AR agonist activity of 7,7'-dihaloDIMs was significantly decreased after 48 hr, and this was due to decreased AR mRNA and AR protein levels, and the latter response was proteasome-

independent. Results of this study demonstrate that the antiandrogenic activity of symmetrical dihaloDIMs was structure-dependent and the 7,7'-dihaloDIMs exhibited partial AR agonist activity, whereas 4,4'-, 5,5'- and 6,6'-dihaloDIMs and DIM were antiandrogens in transactivation assays. The mechanisms of action of ring-substituted DIMs were also structure-dependent since 4,4'- and 5,5'-dihaloDIMs and DIM did not affect AR expression, and 6,6'- and 7,7'-dihaloDIMs induced degradation of AR protein and AR mRNA levels.

In summary, results of this study confirm that DIM and several symmetrical ring-substituted DIM congeners exhibit antiandrogenic activity. In addition, some isomers, notably 7,7'-dichloro- and 7,7'-dibromoDIM also exhibit partial time-dependent androgenic activity in transfection assays, and these results illustrate that subtle changes in the phenyl ring substitution pattern have marked effects on the androgenic activity of the dihaloDIMs. At the concentrations used in this study, the antiandrogenic activity of the 4,4'-dihaloDIMs was not related to inhibition of DHT-induced nuclear translocation of AR. Our results suggest that the antiandrogenic activity of DIM and 4,4'-dihaloDIMs may be complex and involve multiple pathways including inhibition of nuclear AR-dependent transactivation. We also observed that 6,6'-dihaloDIMs and 7,7'-dihaloDIMs decreased AR expression in LNCaP cells, and current studies are investigating the potential clinical importance of these and other effects of ring-substituted DIMs on the growth of prostate cancer cells/tumors in both *in vitro* and *in vivo* models.

REFERENCES

1. Landis, S.H., Murray, T., Bolden, S. and Wingo, P.A. Cancer statistics. *CA. Cancer J. Clin.* 48:6-29, 1998.

2. Shibata, A., Ma, J. and Whittemore, A.S. Prostate cancer incidence and mortality in the United States and the United Kingdom. *J. Natl. Cancer Inst.* 90:1230-1231, 1998.
3. Gao, X., Porter, A.T., Grignon, D.J., Pontes, J.E. and Honn, K.V. Diagnostic and prognostic markers for human prostate cancer. *Prostate* 31:264-281, 1997.
4. Chiarodo, A. National Cancer Institute roundtable on prostate cancer: future research directions. *Cancer Res.* 51:2498-2505, 1991.
5. Sadar, M.D., Hussain, M. and Bruchovsky, N. Prostate cancer: molecular biology of early progression to androgen independence. *Endocr. Relat. Cancer* 6:487-502, 1999.
6. Klotz, L. Hormone therapy for patients with prostate carcinoma. *Cancer* 88:3009-3014, 2000.
7. Morris, M.J. and Scher, H.I. Novel strategies and therapeutics for the treatment of prostate carcinoma. *Cancer* 89:1329-1348, 2000.
8. Boccardo, F. Hormone therapy of prostate cancer: is there a role for antiandrogen monotherapy? *Crit. Rev. Oncol. Hematol.* 35:121-132, 2000.
9. Boasberg, P., Harbaugh, J., Eisenberger, M., Harris, J., Langleben, A. and Ahmann, F. Marimastat in patients with hormone refractory prostate cancer: a dose-finding study. *Proc. Am. Soc. Clin. Oncol.* 16:1126, 1997. (Abstract)
10. Knox, J.D., Bretton, L., Lynch, T., Bowden, G.T. and Nagle, R.B. Synthetic matrix metalloproteinase inhibitor, BB-94, inhibits the invasion of neoplastic human prostate cells in a mouse model. *Prostate* 35:248-254, 1998.
11. Yamaoka, M., Yamamoto, T., Ikeyama, S., Sudo, K. and Fujita, T. Angiogenesis inhibitor TNP-470 (AGM-1470) potently inhibits the tumor growth of hormone-independent human breast and prostate carcinoma cell lines. *Cancer Res.* 53:5233-5236,

- 1993.
12. Folkman, J. Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat. Med.* 1:27-31, 1995.
 13. Folkman, J. Tumor angiogenesis: therapeutic implications. *N. Engl. J. Med.* 285:1182-1186, 1971.
 14. Dorai, T., Olsson, C.A., Katz, A.E. and Buttyan, R. Development of a hammerhead ribozyme against bcl-2. I. Preliminary evaluation of a potential gene therapeutic agent for hormone-refractory human prostate cancer. *Prostate* 32:246-258, 1997.
 15. Pienta, K.J., Nguyen, N.M. and Lehr, J.E. Treatment of prostate cancer in the rat with the synthetic retinoid fenretinide. *Cancer Res.* 53:224-226, 1993.
 16. Pollard, M., Luckert, P.H. and Sporn, M.B. Prevention of primary prostate cancer in Lobund-Wistar rats by N-(4-hydroxyphenyl)retinamide. *Cancer Res.* 51:3610-3611, 1991.
 17. Kelly, W.K., Curley, T., Liebertz, C., Kim, B. and Scher, H.I. Phase II trial of 13-cis-retinoic acid and interferon- α 2a in patients with adenocarcinoma of the prostate. *Proc. Am. Soc. Clin. Oncol.* 15:250, 1996. (Abstract)
 18. Miller, G.J., Stapleton, G.E., Ferrara, J.A., Lucia, M.S., Pfister, Hedlund, T.E. and Upadhy, P. The human prostatic carcinoma cell line LNCaP expresses biologically active, specific receptors for 1 α ,25-dihydroxyvitamin D₃. *Cancer Res.* 52:515-520, 1992.
 19. Miller, G.J., Stapleton, G.E., Hedlund, T.E. and Moffat, K.A. Vitamin D receptor expression, 24-hydroxylase activity, and inhibition of growth by 1 α ,25-dihydroxyvitamin D₃ in seven human prostatic carcinoma cell lines. *Clin. Cancer Res.* 1:997-1003, 1995.

20. Peehl, D.M., Skowronski, R.J., Leung, G.K., Wong, S.T., Stamey, T.A. and Feldman, D. Antiproliferative effects of 1,25-dihydroxyvitamin D3 on primary cultures of human prostatic cells. *Cancer Res.* 54:805-810, 1994.
21. Gross, C., Stamey, T., Hancock, S. and Feldman, D. Treatment of early recurrent prostate cancer with 1,25-dihydroxyvitamin D3 (calcitriol). *J. Urol.* 159:2035-2039, 1998.
22. Kubota, T., Koshizuka, K., Williamson, E.A., Asou, H., Said, J.W., Holden, S., Miyoshi, I. and Koeffler, H.P. Ligand for peroxisome proliferator-activated receptor γ (troglitazone) has potent antitumor effect against human prostate cancer both *in vitro* and *in vivo*. *Cancer Res.* 58:3344-3352, 1998.
23. Tontonoz, P., Singer, S., Forman, B.M., Sarraf, P., Fletcher, J.A., Fletcher, C.D., Brun, R.P., Mueller, E., Altiock, S., Oppenheim, H., Evans, R.M. and Spiegelman, B.M. Terminal differentiation of human liposarcoma cells induced by ligands for peroxisome proliferator-activated receptor γ and the retinoid X receptor. *Proc. Natl. Acad. Sci. U. S. A.* 94:237-241, 1997.
24. Tontonoz, P., Hu, E. and Spiegelman, B.M. Stimulation of adipogenesis in fibroblasts by PPAR γ 2, a lipid-activated transcription factor. *Cell* 79:1147-1156, 1994.
25. Mueller, E., Sarraf, P., Tontonoz, P., Evans, RM, Martin, K.J., Zhang, M., Fletcher, C., Singer, S. and Spiegelman, B.M. Terminal differentiation of human breast cancer through PPAR γ . *Mol. Cell* 1:465-470, 1998.
26. Smith, M., Mueller, E., Demetri, G., Kaufman, D., Oh, W. and Jacobsen, J. Preliminary results: phase II trial of troglitazone for androgen-dependent (AD) and androgen-independent (AI) prostate cancer. *Proc. Am. Soc. Clin. Oncol.* 18:328, 1999. (Abstract)

27. Jana, N.R., Sarkar, S., Ishizuka, M., Yonemoto, J., Tohyama, C. and Sone, H. Cross-talk between 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and testosterone signal transduction pathways in LNCaP prostate cancer cells. *Biochem. Biophys. Res. Commun.* 256:462-468, 1999.
28. Safe, S., Qin, C. and McDougal, A. Development of selective aryl hydrocarbon receptor modulators (SARMs) for treatment of breast cancer. *Expert Opinion on Investigational Drugs* 8:1385-1396, 1999.
29. Dickerson, R., Howie-Keller, L. and Safe, S. Alkyl polychlorinated dibenzofurans and related compounds as antiestrogens in the female rat uterus: structure-activity studies. *Toxicol. Appl. Pharmacol.* 135:287-298, 1995.
30. McDougal, A., Wilson, C. and 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. McDougal, A. *Aromatic Hydrocarbon Receptor-Based Antiestrogens as Treatments for Breast Cancer*. Texas A&M University, Ph.D. Dissertation, 2000.
32. Safe, S., Wargovich, M.J., Lamartinierre, C.A. and Mukhtar, H. Symposium on mechanism of action of naturally-occurring anticarcinogens. *Toxicol. Sci.* 5:1-8, 1999.
33. 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.
34. Chen, I., McDougal, A., Wang, F. and Safe, S. Aryl hydrocarbon receptor-mediated antiestrogenic and antitumorigenic activity of diindolylmethane. *Carcinogenesis* 19:1631-1639, 1998.

35. McDougal, A., Sethi-Gupta, M., Ramamoorthy, K., Sun, G. and 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.
36. Koliopanos, A., Kleeff, J., Xiao, Y., Safe, S., Zimmerman, A., Buchler, M.W. and Friess, H. Increased aryl hydrocarbon receptor expression offers a potential therapeutic target in pancreatic cancer. *Oncogene* 21:6059-6070, 2002.
37. Alarid, E.T., Bakopoulos, N. and Solodin, N. Proteasome-mediated proteolysis of estrogen receptor: a novel component in autologous down-regulation. *Mol. Endocrinol.* 13:1522-1534, 1999.
38. Nawaz, Z., Lonard, D.M., Dennis, A.P., Smith, C.L. and O'Malley, B.W. Proteasome-dependent degradation of the human estrogen receptor. *Proc. Natl. Acad. Sci. U. S. A.* 96:1858-1862, 1999.
39. El Khissiin, A. and Leclercq, G. Implication of proteasome in estrogen receptor degradation. *FEBS Lett.* 448:160-166, 1999.
40. Lonard, D.M., Nawaz, Z., Smith, C.L. and O'Malley, B.W. The 26S proteasome is required for estrogen receptor- α and coactivator turnover and for efficient estrogen receptor- α transactivation. *Mol. Cell* 5:939-948, 2000.
41. Roberts, B.J. and Whitelaw, M.L. Degradation of the basic helix-loop-helix/Per-ARNT-Sim homology domain dioxin receptor via the ubiquitin/proteasome pathway. *J. Biol. Chem.* 274:36351-36356, 1999.
42. Davarinos, N.A. and Pollenz, R.S. Aryl hydrocarbon receptor imported into the nucleus following ligand binding is rapidly degraded via the cytoplasmic proteasome following

- nuclear export. *J. Biol. Chem.* 274:28708-28715, 1999.
43. Ma, Q. and Baldwin, K.T. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin-induced degradation of aryl hydrocarbon receptor (AhR) by the ubiquitin-proteasome pathway. Role of the transcription activation and DNA binding of AhR. *J. Biol. Chem.* 275:8432-8438, 2000.
 44. Boudjelal, M., Wang, Z., Voorhees, J.J. and Fisher, G.J. Ubiquitin/proteasome pathway regulates levels of retinoic acid receptor γ and retinoid X receptor α in human keratinocytes. *Cancer Res.* 60:2247-2252, 2000.
 45. Kopf, E., Plassat, J.-L., Vivat, V., de Thé, H., Chambon, P. and Rochette-Egly, C. Dimerization with retinoid X receptors and phosphorylation modulate the retinoic acid-induced degradation of retinoic acid receptors α and γ through the ubiquitin-proteasome pathway. *J. Biol. Chem.* 275:33280-33288, 2000.
 46. Dace, A., Zhao, L., Park, K.S., Furuno, T., Takamura, N., Nakanishi, M., West, B.L., Hanover, J.A. and Cheng, S. Hormone binding induces rapid proteasome-mediated degradation of thyroid hormone receptors. *Proc. Natl. Acad. Sci. U. S. A.* 97:8985-8990, 2000.
 47. Sheflin, L., Keegan, B., Zhang, W. and Spaulding, S.W. Inhibiting proteasomes in human HepG2 and LNCaP cells increases endogenous androgen receptor levels. *Biochem. Biophys. Res. Commun.* 275:144-150, 2000.
 48. Le, H.T., Schaldach, C.M., Firestone, G.L. and Bjeldanes, L.F. Plant-derived 3,3'-diindolylmethane is a strong androgen antagonist in human prostate cancer cells. *J. Biol. Chem.* 278:21136-21145, 2003.
 49. van Bokhoven, A., Varella-Garcia, M., Korch, C., Johannes, W.U., Smith, E.E., Miller, H.L., Nordeen, S.K., Miller, G.J. and Lucia, M.S. Molecular characterization of human

- prostate carcinoma cell lines. *Prostate* 57:205-225, 2003.
50. McDonald, S., Brive, L., Agus, D.B., Scher, H.I. and Ely, K.R. Ligand responsiveness in human prostate cancer: structural analysis of mutant androgen receptors from LNCaP and CWR22 tumors. *Cancer Res.* 60:2317-2322, 2000.
 51. Ren, F., Zhang, S., Mitchell, S.H., Butler, R. and Young, C.Y. Tea polyphenols down-regulate the expression of the androgen receptor in LNCaP prostate cancer cells. *Oncogene* 19:1924-1932, 2000.
 52. Cha, T.L., Qiu, L., Chen, C.T., Wen, Y. and Hung, M.C. Emodin down-regulates androgen receptor and inhibits prostate cancer cell growth. *Cancer Res.* 65:2287-2295, 2005.
 53. Blanchere, M., Berthaut, I., Portois, M.C., Mestayer, C. and Mowszowicz, I. Hormonal regulation of the androgen receptor expression in human prostatic cells in culture. *J. Steroid Biochem. Mol. Biol.* 66:319-326, 1998.
 54. Furutani, T., Watanabe, T., Tanimoto, K., Hashimoto, T., Koutoku, H., Kudoh, M., Shimizu, Y., Kato, S. and Shikama, H. Stabilization of androgen receptor protein is induced by agonist, not by antagonists. *Biochem. Biophys. Res. Commun.* 294:779-784, 2002.
 55. Velasco, A.M., Gillis, K.A., Li, Y., Brown, E.L., Sadler, T.M., Achilleos, M., Greenberger, L.M., Frost, P., Bai, W. and Zhang, Y. Identification and validation of novel androgen-regulated genes in prostate cancer. *Endocrinology* 145:3913-3924, 2004.

APPENDIX

- Safe, S. and McDougal, A. Mechanism of action and development of selective aryl hydrocarbon receptor modulators for treatment of hormone-dependent cancers. *Int. J. Oncol.* 20:1123-1128, 2002.
- Morrow, D., McDougal, A. and Safe, S. Comparative aryl hydrocarbon receptor-hormone receptor crosstalk in breast and prostate cancer cells. Society of Toxicology Annual Meeting, Nashville, TN. March, 2002. Abstract #LB31.
- Morrow, D., Qin, C., Smith III, R. and Safe, S. Aryl hydrocarbon receptor-mediated inhibition of LNCaP prostate cancer cell growth and hormone-induced transactivation. *J. Steroid Biochem. Mol. Biol.* 88:27-36, 2004.
- Morrow, D. and Safe, S. Aryl hydrocarbon receptor agonists inhibit hormone-induced transactivation in prostate cancer cells. Society of Toxicology Annual Meeting, Salt Lake City, UT, 2003. Abstract #1785.
- Kotha, L. and Safe, S. Structure-dependent androgen receptor agonist/antagonist activities and AR downregulation by dichloro- and dibromo-substituted 1,1-bis(3'-indolyl)methanes. *Mol. Carcinog.* submitted, 2006.

Mechanism of action and development of selective aryl hydrocarbon receptor modulators for treatment of hormone-dependent cancers (Review)

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Abstract. Ligand-activated receptors are extensively used as targets for developing tissue-selective drugs for treatment of multiple diseases including cancers. The aryl hydrocarbon receptor (AhR) is a basic helix-loop-helix transcription factor that binds both synthetic chemicals such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and naturally-occurring phytochemicals, sterols and heme breakdown products. The high affinity ligand TCDD induces several AhR-mediated changes in gene expression, tissue/species-specific toxicities, and both tumorigenic and anticarcinogenic responses including inhibition of estrogen-dependent mammary and uterine tumor formation and growth. Research in this laboratory has demonstrated that TCDD inhibits E2-induced responses in the rodent uterus and mammary tumors (growth inhibition) and in breast and endometrial cancer cell lines through complex inhibitory AhR-estrogen receptor (ER) crosstalk. 6-Alkyl-1,3,8-trichlorodibenzofurans and substituted diindolyl-methanes represent two structural classes of selective AhR modulators (SAhRMs). These compounds are relatively non-toxic and inhibit ER-positive and ER-negative mammary tumor growth, and synergize with tamoxifen to inhibit breast cancer growth and block tamoxifen-induced estrogenic activity in the uterus. Preliminary studies also indicate that SAhRMs inhibit prostate cancer cell growth, and there is evidence for inhibitory AhR-androgen receptor crosstalk. SAhRMs represent a novel class of drugs for treatment of hormone-dependent cancers, and combined therapies of SAhRMs with tamoxifen and other selective ER modulators (SERMs) provides a new approach for treating women with breast cancer.

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1. Introduction

The aryl hydrocarbon receptor (AhR) is a member of the basic helix-loop-helix family of nuclear transcription factors, and this receptor was initially identified by its high affinity binding to the environmental toxicant, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (1,2). TCDD and structurally-related halogenated aromatics modulate expression of multiple genes which play a role in the species-, sex- and age-specific toxic, genotoxic and anticarcinogenic responses associated with exposure of laboratory animals to these compounds (2-6). The endogenous ligand for the AhR is unknown; however, several reports have now demonstrated that this receptor binds structurally-diverse synthetic chemicals as well as aromatic amines in cooked foods, phytochemicals including indole-3-carbinol (I3C) and related indole-derived compounds, carotenoids, flavonoids, steroidal compounds, and bilirubin (Fig. 1) (7-14). Interestingly, steroid hormone receptors and other ligand-activated nuclear receptors also bind different structural classes of chemicals and many of these receptors are targets for developing drugs that selectively ameliorate one or more receptor-mediated responses. For example, selective estrogen receptor (ER) modulators have been developed as both tissue-specific ER agonists and antagonists for treating breast cancer and various postmenopausal symptoms in women (15). Since the ligand-activated AhR modulates diverse genes/responses, this receptor is an excellent target for drug development. For example, TCDD inhibits spontaneous 17 β -estradiol (E2)-induced mammary and uterine tumor formation in Sprague-Dawley rats suggesting that TCDD exhibits antiestrogenic activity. Research in our

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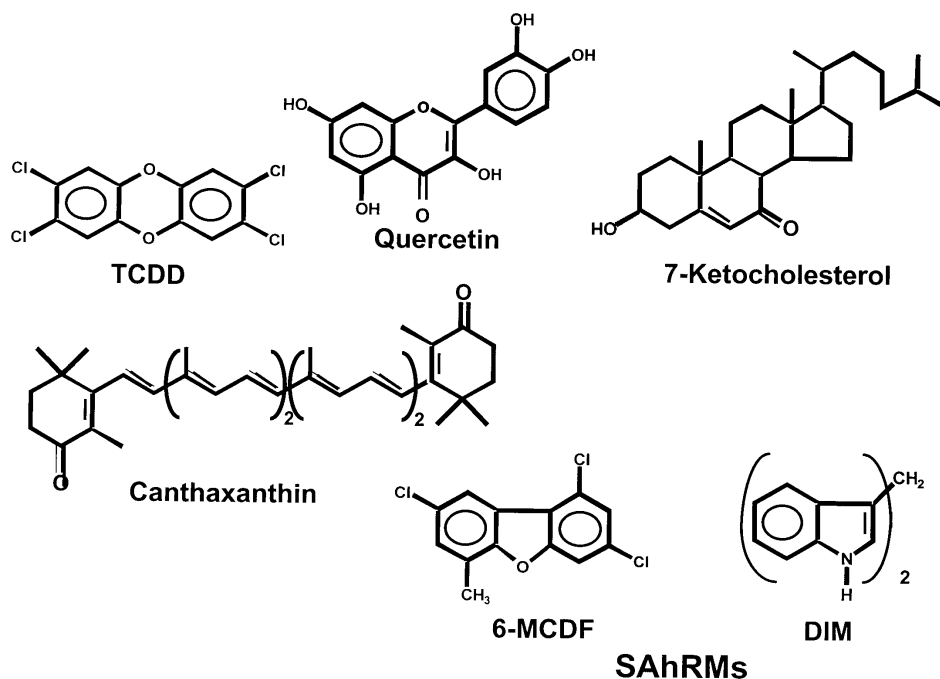


Figure 1. Structurally diverse compounds that bind the Ah receptor including the SAhRMs 6-MCDF and DIM.

laboratory has focused on studying the mechanisms of inhibitory AhR-ER crosstalk (3,16-18) and developing selective AhR modulators (SAhRMs) for treating breast and endometrial cancers (19,20).

2. Inhibitory AhR-ER α crosstalk: *in vivo*

The antiestrogenic/antitumorigenic activity of TCDD observed in female Sprague-Dawley rats stimulated *in vivo* and *in vitro* studies to determine the specificity of this response (reviewed in refs. 13,16-20). The immature and/or ovariectomized female rat and mouse uterus has been used as a model, and TCDD inhibited several E2-induced uterine responses including progesterone receptor (PR) binding, uterine wet weight, peroxidase activity, *c-fos* and epidermal growth factor receptor (EGFR) mRNA levels, and EGFR binding. Similar results were obtained for the growth of E2-dependent mammary tumors in carcinogen-induced Sprague-Dawley rats and athymic nude mice bearing breast cancer cell xenografts. Recent studies (21) also show that women accidentally exposed to TCDD in Seveso, Italy in 1976 exhibit decreased rates of breast and endometrial cancer, and these observations in humans parallel the effects of TCDD in rodents.

3. Inhibitory AhR-ER α crosstalk: *in vitro*

The interactions of the AhR and ER signaling pathways have been investigated in several ER-positive breast and endometrial cancer cell lines, and these include MCF7, ZR-75 and T47D breast cancer cell lines and Ishikawa, HEC1A and ECC-1 endometrial cancer cells. All of these cell lines express the AhR, and AhR agonists such as TCDD induce CYP1A1 gene expression, a highly characteristic Ah-responsive gene in cell

culture and animal models. In addition, TCDD and related compounds also inhibited E2-induced breast and endometrial cancer cell proliferation (22-25). Subsequent studies have demonstrated that AhR agonists inhibit several E2-induced responses at the gene, protein and reporter gene level using constructs containing E2-responsive gene promoter inserts. For example, in MCF-7 human breast cancer cells, TCDD inhibits E2-induced PR, pS2, cathepsin D mRNA and protein levels, *c-fos* and prolactin mRNA levels, cyclin D1 protein, retinoblastoma (Rb) protein phosphorylation, glucose metabolism, and plasminogen activator activity. A major pathway for AhR-mediated inhibition of E2-induced MCF-7 cell proliferation is linked to the selective inhibition of critical E2-induced cell cycle regulatory proteins such as cyclin D1, Rb phosphorylation and E2F1 (Fig. 2), and these inhibitory responses block E2-induced G₁-S phase cell cycle progression (26).

Inhibitory AhR-ER crosstalk is observed not only in breast cancer cells, but also in E2-responsive endometrial and ovarian cancer cells where TCDD inhibits E2-induced cell proliferation (24,25,27). The mechanisms associated with the effects of AhR agonists on hormone-induced cell proliferation have primarily been investigated in breast cancer cell lines (see below); however, it is likely that interactions between AhR-ER signaling pathways are comparable in breast, endometrial and ovarian cell lines.

4. Mechanisms of AhR-ER crosstalk

Results of initial studies in this laboratory showed that TCDD inhibited E2-induced expression of multiple genes and similar results were observed in transient transfection studies using constructs containing E2-responsive gene promoter inserts. These results suggested that the ligand-bound AhR disrupted

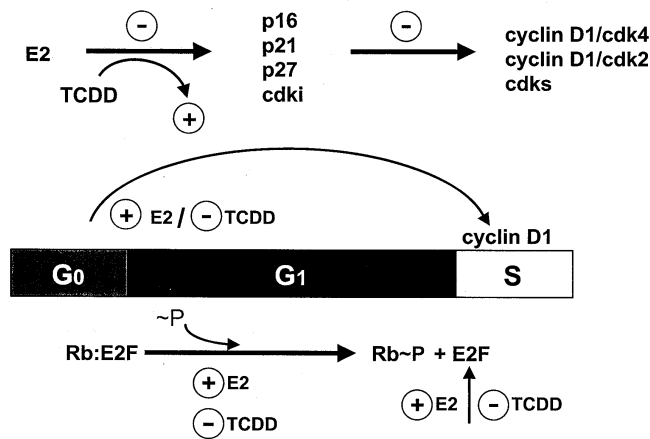


Figure 2. Interactions of E2 and TCDD on cell cycle progression in MCF-7 human breast cancer cells (26).

a promoter- and E2-dependent transcriptionally-active complex. This type of inhibitory crosstalk could occur via several pathways including AhR-dependent induction or inhibition of a critical factor involved in hormone-induced transactivation, direct interaction of the AhR with critical *trans*-acting factors or associated proteins, and interaction of the AhR with critical *cis*-acting elements (Fig. 3). Analysis of the E2-responsive cathepsin D, *c-fos*, pS2 and heat shock protein 27 gene promoters have identified pentanucleotide GCGTG sequences which are required for inhibitory AhR-ER crosstalk (28-32), and these motifs weakly bind the AhR complex and correspond to the core of a dioxin response element (DRE). Inhibitory DREs (iDRE) are strategically located within gene promoters and block formation of a transcriptionally-active complex. For example, binding of the AhR complex to the upstream iDRE in the cathepsin D gene promoter (-175 to -181) disrupts formation of the ER/Sp1 complex which is also formed in the same region of the promoter (-199 to -165). Results of more recent studies demonstrate that several constructs containing E2-responsive gene promoter (e.g. retinoic acid receptor α 1) inserts are also inhibited by AhR agonists in transient transfection studies, and these promoters do not contain functional iDREs. Currently, we are investigating other mechanisms including the role of AhR-activated proteasome-dependent degradation of the ER (33) and sequestration of the ER through direct AhR-ER interactions.

5. Selective AhR modulators (SAhRMs) for treatment of ER-positive breast cancer

The environmental toxicant TCDD has been routinely used as a prototype for investigating AhR-mediated responses including inhibitory AhR-ER interactions in the rodent uterus and mammary tumors and in breast/endometrial cancer cells. Alternate substituted 6-alkyl-1,3,8-trichlorodibenzofurans were initially characterized as AhR antagonists which exhibited low toxicity, and in combination with TCDD, one of these analogs [6-methyl-1,3,8-trichlorodibenzofuran (6-MCDF)] (Fig. 1) inhibited TCDD-induced CYP1A1 gene

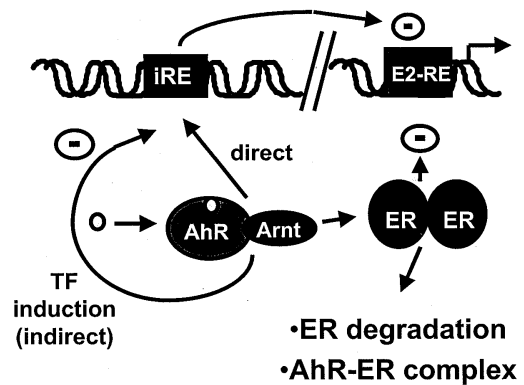


Figure 3. Inhibitory AhR-ER α crosstalk. Inhibitory responses are gene-specific and may involve direct interactions with iDREs (28-32), proteasome-dependent degradation of ER α (33), or induction of inhibitory transcription factors (TFs).

expression, immunotoxicity, hepatic porphyria, and cleft palate in mice (34-37). However, 6-MCDF and related compounds were agonists for inhibitory AhR-ER crosstalk in both *in vitro* and *in vivo* models (38-41). For example, 6-MCDF inhibits carcinogen-induced mammary tumor growth in female Sprague-Dawley at doses as low as 50 μ g/kg per day (42,43). Moreover, in ovariectomized female rats of a comparable age, 6-MCDF inhibited tamoxifen-induced estrogenic responses in the uterus (e.g. peroxidase activity, progesterone receptor binding) but did not affect the ER agonist effects of tamoxifen on bone growth (43). These results, coupled with the observed inhibitory AhR-ER crosstalk in endometrial cancer cells suggest that combined therapy with SAhRMs, such as 6-MCDF plus tamoxifen, will be highly effective for treating mammary cancer and also protecting against tamoxifen-induced estrogenic responses in the uterus. This latter interaction is important since long-term treatment with tamoxifen is associated with an increased incidence of endometrial cancer (44). We have also investigated the AhR agonist activities of diindolylmethane (DIM) (Fig. 1) and a series of dihalo- and dialkylDIM analogs (45-47). These compounds bind the AhR and exhibit some of the agonist/antagonist activities observed for 6-MCDF. Moreover, in rodent models for mammary carcinogenesis, many of the DIM compounds were potent inhibitors of mammary tumor growth.

6. SAhRMs for treatment of ER-negative breast cancer

The AhR is expressed in ER-positive and -negative breast cancer cell lines and in human mammary tumors; however, initial studies indicated that the AhR was not functional in ER-negative cell lines and the diagnostic induction of CYP1A1-dependent activity by TCDD was not observed (48,49). This failure to observe induction could be overcome by transient overexpression of ER α (50,51), and a recent study suggested that Ah non-responsiveness in ER-negative MDA-MB-231 cells may be linked to overexpression of heat shock protein 90 which sequesters and inactivates the AhR (52). ER-negative MDA-MB-468 express a functional AhR

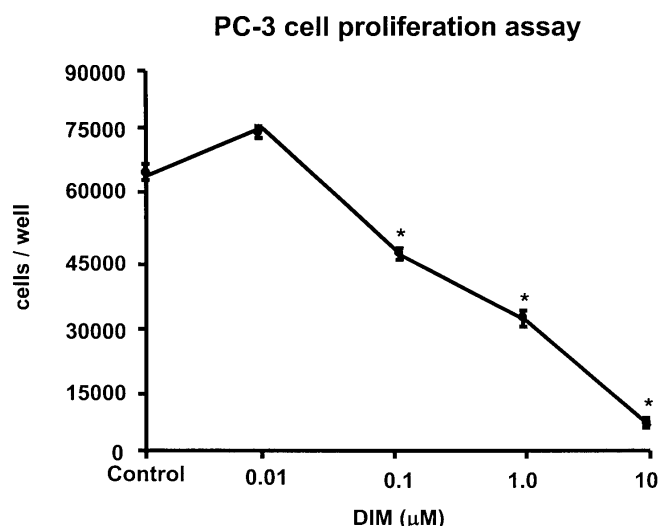


Figure 4. Inhibition of PC3 prostate cancer cell growth by different concentrations of DIM.

and TCDD induces CYP1A1 gene expression; moreover, both TCDD and 6-MCDF inhibit MDA-MB-468 cell growth through induction of transforming growth factor α which is growth inhibitory in this cell line (53). Results of ongoing studies also indicate that 6-MCDF inhibits tumor growth in athymic nude mice bearing MCF-7 or MDA-MB-468 cell xenografts. In addition, a more extensive survey of ER-negative breast cancer cells shows that several of these cell lines, including MDA-MB-453, MDA-MB-435, HCC-38 and BT-20 cells, express a functional AhR (i.e. CYP1A1 inducibility by TCDD) and 6-MCDF inhibits cell proliferation. Current studies are further characterizing the growth inhibitory and antitumorigenic activity of 6-MCDF and related SAhRMs using ER-negative breast cancer cells and thereby expanding the potential therapeutic applications of SAhRMs for treating breast cancer.

7. Potential applications of SAhRMs for treating other cancers

The AhR is expressed in cancer cells derived from tumors from multiple tissues; however, the potential applications of SAhRMs for inhibiting growth of these tumors has not been extensively investigated. Research in this laboratory has demonstrated that TCDD and/or 6-MCDF inhibit E2-induced proliferation of E2-responsive PE04 ovarian and Ishikawa/ECC1 endometrial cancer cell lines (24,25,27). Smoking is known to be protective for development of endometrial cancer in women and benzo[a]pyrene, an AhR agonist that is a component of cigarette smoke and other combustion products also inhibits E2-induced proliferation of Ishikawa endometrial cancer cells (25,54,55). Recent studies show that AhR agonists also block androgen receptor (AR) signaling including testosterone-induced prostate-specific antigen (protein and mRNA) (56,57). We have also investigated inhibitory AhR-AR interactions in prostate cancer cells and the results in Fig. 4 demonstrate that DIM inhibits proliferation of PC-3 prostate cancer cells maintained in 0

and 1% serum. These results suggest that SAhRMs may also be useful for inhibiting prostate and possibly other cancers, and the applications and mechanisms of action of these compounds are currently being investigated.

8. Summary

Ligand-activated receptors are ideal targets for developing tissue-selective modulators for treating different diseases. The AhR is widely expressed in mammalian tissues and tumors, and it is clear from studies on TCDD that multiple genes/responses are mediated through the AhR, and these include inhibition or enhancement of immune responses, reproductive toxicity, a wasting syndrome, carcinogenic and anticarcinogenic responses, proteasome activation, and tissue-specific up- or downregulation of several genes. Our research has focused on development of tissue-selective SAhRMs for treatment of mammary cancer and it is possible that SAhRMs may also inhibit growth of tumors in other tissues, and these are currently being investigated.

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References

- Poland A, Glover E and Kende AS: Stereospecific, high affinity binding of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin by hepatic cytosol: evidence that the binding species is receptor for induction of aryl hydrocarbon hydroxylase. *J Biol Chem* 251: 4936-4946, 1976.
- Poland A and Knutson JC: 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin and related halogenated aromatic hydrocarbons. Examinations of the mechanism of toxicity. *Annu Rev Pharmacol Toxicol* 22: 517-554, 1982.
- Safe S: Modulation of gene expression and endocrine response pathways by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and related compounds. *Pharmacol Ther* 67: 247-281, 1995.
- Wilson CL and Safe S: Mechanisms of ligand-induced aryl hydrocarbon receptor-mediated biochemical and toxic responses. *Toxicol Pathol* 26: 657-671, 1998.
- Swanson HI and Bradfield CA: The Ah-receptor: genetics, structure and function. *Pharmacogenetics* 3: 213-223, 1993.
- Whitlock JP Jr: Mechanistic aspects of dioxin action. *Chem Res Toxicol* 6: 754-763, 1993.
- Denison MS, Seidel SD, Rogers WJ, Ziccardi M, Winter GM and Heath-Pagliuso S: Natural and synthetic ligands for the Ah receptor. In: *Molecular Biology Approaches to Toxicology*. Puga A and Kendall RJ (eds.) Taylor and Francis, London, pp3-33, 1998.
- Gradelet S, Astorg P, Pineau T, Canivenc MC, Siess MH, Leclerc J and 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.
- Bjeldanes LF, Kim JY, Grose KR, Bartholomew JC and 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.
- Chen I, Safe S and 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.

11. Quadri SA, Qadri AN, Hahn ME, Mann KK and Sherr DH: The bioflavonoid galangin blocks aryl hydrocarbon receptor activation and polycyclic aromatic hydrocarbon-induced pre-B cell apoptosis. *Mol Pharmacol* 58: 515-525, 2000.
12. Savouret JF, Antenos M, Quesne M, Xu J, Milgrom E and Casper RF: 7-Ketocholesterol is an endogenous modulator for the arylhydrocarbon receptor. *J Biol Chem* 276: 3054-3059, 2001.
13. Chun YJ, Ryu SY, Jeong TC and Kim MY: Mechanism-based inhibition of human cytochrome P450 1A1 by rhapontigenin. *Drug Metab Dispos* 29: 389-393, 2001.
14. Sinal CJ and Bend JR: Aryl hydrocarbon receptor-dependent induction of Cyp1a1 by bilirubin in mouse hepatoma Hepa 1c1c7 cells. *Mol Pharmacol* 52: 590-599, 1997.
15. Fuqua SA, Russo J, Shackney SE and Stearns ME: Estrogen, estrogen receptors and selective estrogen receptor modulators in human breast cancer. *J Women's Cancer* 2: 21-32, 2000.
16. Zacharewski T and Safe S: Antiestrogenic activity of TCDD and related compounds. In: *Reproductive and Developmental Toxicology*. Korach KS (ed.) Marcel Dekker, Inc., New York, pp431-448, 1998.
17. Porter W and Safe S: Estrogenic and antiestrogenic compounds. In: *Molecular Biology Approaches to Toxicology*. Puga A and Wallace KB (eds.) Techbooks, Fairfax, VA, pp267-283, 1998.
18. Safe S: 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and related environmental antiestrogens: characterization and mechanism of action. In: *Endocrine Disruptors*. Naz RK (ed.) CRC Press, Boca Raton, FL, pp187-221, 1999.
19. Safe S, Qin C and McDougal A: Development of selective aryl hydrocarbon receptor modulators (SARMs) for treatment of breast cancer. *Expert Opin Invest Drugs* 8: 1385-1396, 1999.
20. Safe S, McDougal A, Gupta MS 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* 3: 37-45, 2001.
21. Bertazzi PA, Consonni D, Bachetti S, Rubagotti M, Baccarelli A, Zocchetti C and Pesatori AC: Health effects of dioxin exposure: a 20-year mortality study. *Am J Epidemiol* 153: 1031-1044, 2001.
22. Biegel L and Safe S: Effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on cell growth and the secretion of the estrogen-induced 34-, 52- and 160-kDa proteins in human breast cancer cells. *J Steroid Biochem Mol Biol* 37: 725-732, 1990.
23. Fernandez P and Safe S: Growth inhibitory and antimutagenic activity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in T47D human breast cancer cells. *Toxicol Lett* 61: 185-197, 1992.
24. Castro-Rivera E, Wormke M and Safe S: Estrogen and aryl hydrocarbon responsiveness of ECC-1 endometrial cancer cells. *Mol Cell Endocrinol* 150: 11-21, 1999.
25. Wormke M, Castro-Rivera E, Chen I and Safe S: Estrogen and aryl hydrocarbon receptor expression and crosstalk in human Ishikawa endometrial cancer cells. *J Steroid Biochem Mol Biol* 72: 197-207, 2000.
26. Wang W, Smith R and Safe S: Aryl hydrocarbon receptor-mediated antiestrogenicity in MCF-7 cells: modulation of hormone-induced cell cycle enzymes. *Arch Biochem Biophys* 356: 239-248, 1998.
27. Rowlands C, Krishnan V, Wang X, Santostefano M, Safe S, Miller WR and Langdon S: Characterization of the aryl hydrocarbon (Ah) receptor and Ah-responsiveness in human ovarian carcinoma cell lines. *Cancer Res* 53: 1802-1807, 1993.
28. Krishnan V, Porter W, Santostefano M, Wang X and Safe S: Molecular mechanism of inhibition of estrogen-induced cathepsin D gene expression by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in MCF-7 cells. *Mol Cell Biol* 15: 6710-6719, 1995.
29. Duan R, Porter W, Samudio I, Vyhldal C, Kladde M and Safe S: Transcriptional activation of c-fos protooncogene by 17 β -estradiol: mechanism of aryl hydrocarbon receptor-mediated inhibition. *Mol Endocrinol* 13: 1511-1521, 1999.
30. Moore M, Wang X, Lu YF, Wormke M, Craig A, Gerlach JH, Burghardt R, Barhoumi R and Safe S: Benzo[a]pyrene-resistant MCF-7 human breast cancer cells. A unique aryl hydrocarbon-nonresponsive clone. *J Biol Chem* 269: 11751-11759, 1994.
31. Gillesby B, Santostefano M, Porter W, Wu ZF, Safe S and Zacharewski T: Identification of a motif within the 5'-regulatory region on pS2 which is responsible for Ap1 binding and TCDD-mediated suppression. *Biochemistry* 36: 6080-6089, 1997.
32. Porter W, Wang F, Duan R, Qin C, Castro-Rivera E and Safe S: Transcriptional activation of heat shock protein 27 gene expression by 17 β -estradiol and modulation by antiestrogens and aryl hydrocarbon receptor agonists: estrogenic activity of ICI 164,384. *J Mol Endocrinol* 26: 31-42, 2001.
33. Harris M, Zacharewski T, Astroff B and Safe S: Partial antagonism of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-mediated induction of aryl hydrocarbon hydroxylase by 6-methyl-1,3,8-trichlorodibenzofuran: mechanistic studies. *Mol Pharmacol* 35: 729-735, 1989.
34. Wormke M, Stoner M, Saville B and Safe S: Crosstalk between estrogen receptor α and the aryl hydrocarbon receptor in breast cancer cells involves unidirectional activation of proteasomes. *FEBS Lett* 478: 109-112, 2000.
35. Astroff B, Zacharewski T, Safe S, Arlotto MP, Parkinson A, Thomas P and Levin W: 6-Methyl-1,3,8-trichlorodibenzofuran as a 2,3,7,8-tetrachlorodibenzo-*p*-dioxin antagonist: inhibition of the induction of rat cytochrome P-450 isozymes and related monooxygenase activities. *Mol Pharmacol* 33: 231-236, 1988.
36. Bannister R, Biegel L, Davis D, Astroff B and Safe S: 6-Methyl-1,3,8-trichlorodibenzofuran (MCDF) as a 2,3,7,8-tetrachlorodibenzo-*p*-dioxin antagonist in C57BL/6 mice. *Toxicology* 54: 139-150, 1989.
37. Yao C and Safe S: 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin-induced porphyria in genetically inbred mice: partial antagonism and mechanistic studies. *Toxicol Appl Pharmacol* 100: 208-216, 1989.
38. Astroff B and 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.
39. Astroff B and Safe S: 6-Alkyl-1,3,8-trichlorodibenzofurans as antiestrogens in female Sprague-Dawley rats. *Toxicology* 69: 187-197, 1991.
40. Zacharewski T, Harris M, Biegel L, Morrison V, Merchant M and Safe S: 6-Methyl-1,3,8-trichlorodibenzofuran (MCDF) as an antiestrogen in human and rodent cancer cell lines: evidence for the role of the Ah receptor. *Toxicol Appl Pharmacol* 13: 311-318, 1992.
41. Dickerson R, Howie-Keller L and Safe S: Alkyl polychlorinated dibenzofurans and related compounds as antiestrogens in the female rat uterus: structure-activity studies. *Toxicol Appl Pharmacol* 135: 287-298, 1995.
42. McDougal A, Wilson C and 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.
43. McDougal A: *Aromatic Hydrocarbon Receptor-Based Antiestrogens as Treatments for Breast Cancer*. Texas A&M University. Thesis/Dissertation, 2000.
44. Bernstein L, Deapen D, Cerhan JR, Schwartz SM, Liff J, McGann-Maloney E, Perlman JA and Ford L: Tamoxifen therapy for breast cancer and endometrial cancer risk. *J Natl Cancer Inst* 91: 1654-1662, 1999.
45. Chen I, McDougal A, Wang F and Safe S: Aryl hydrocarbon receptor-mediated antiestrogenic and antitumorigenic activity of diindolylmethane. *Carcinogenesis* 19: 1631-1639, 1998.
46. McDougal A, Sethi-Gupta M, Ramamoorthy K, Sun G and Safe S: Inhibition of carcinogen-induced rat mammary tumor growth and other estrogen-dependent responses by symmetrical dihalo-substituted analogs of diindolylmethane. *Cancer Lett* 151: 169-179, 2000.
47. McDougal A, Gupta MS, 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.
48. Vickers PJ, Dufresne MJ and Cowan KH: Relation between cytochrome P4501A1 expression and estrogen receptor content of human breast cancer cells. *Mol Endocrinol* 3: 157-164, 1989.
49. Wang X, Thomsen JS, Santostefano M, Rosengren R, Safe S and Perdev GH: Comparative properties of the nuclear Ah receptor complex from several human cell lines. *Eur J Pharmacol* 293: 191-205, 1995.
50. Mattson MP and Kater SB: Isolated hippocampal neurons in cryopreserved long-term cultures: development of neuroarchitecture and sensitivity to NMDA. *Int J Dev Neurosci* 6: 439-452, 1988.
51. Wang WL, Thomsen JS, Porter W, Moore M and Safe S: Effect of transient expression of the oestrogen receptor on constitutive and inducible CYP1A1 in Hs578T human breast cancer cells. *Br J Cancer* 73: 316-322, 1996.

52. Caruso JA, Laird DW and Batist G: Role of HSP90 in mediating cross-talk between the estrogen receptor and the Ah receptor signal transduction pathways. *Biochem Pharmacol* 58: 1395-1403, 1999.
53. Wang W, Porter W, Burghardt R and Safe S: Mechanism of inhibition of MDA-MB-468 breast cancer cell growth by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Carcinogenesis* 18: 925-933, 1997.
54. Lesko SM, Rosenberg L, Kaufman DW, Helmrigh SP, Miller DR, Strom B, Schottenfeld D, Rosenschein NB, Knapp RC, Lewis J and Shapiro S: Cigarette smoking and the risk of endometrial cancer. *N Engl J Med* 313: 593-596, 1985.
55. Baron JA, La Vecchia C and Levi F: The antiestrogenic effect of cigarette smoking in women. *Am J Obstet Gynecol* 162: 502-514, 1990.
56. Jana NR, Sarkar S, Ishizuka M, Yonemoto J, Tohyama C and Sone H: Cross-talk between 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and testosterone signal transduction pathways in LNCaP prostate cancer cells. *Biochem Biophys Res Commun* 256: 462-468, 1999.
57. Jana NR, Sarkar S, Ishizuka M, Yonemoto J, Tohyama C and Sone H: Comparative effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on MCF-7, RL95-2, and LNCaP cells: role of target steroid hormones in cellular responsiveness to CYP1A1 induction. *Mol Cell Biol Res Commun* 4: 174-180, 2000.



den Berg et al. 1998). A risk assessment was conducted to identify a concentration of PCDD/Fs in soils that would not pose an unacceptable health risk to residents (i.e., a site-specific soil criterion). US Environmental Protection Agency (EPA) and Michigan Department of Environmental Quality (MDEQ) guidance for conducting residential human health risk assessments was used for the assessment with exposures *via* soil ingestion and dermal contact assessed. All other potential exposure pathways were considered to be *de minimus*. A probabilistic analysis was used to calculate the soil criterion for a theoretical increased risk of 1×10^5 (1 in 100,000) using the MDEQ's cancer slope factor of 49,000 (mg/kg-day)¹ for PCDD/Fs (MDEQ 2001). Probability density functions (PDFs) were used to describe child soil ingestion rates, body weights, skin surface areas, soil adherence rates, and exposure durations. All other parameters were represented by point estimate values. The results of a site-specific *in vitro* study of the bioaccessibility of PCDD/Fs in site soils were incorporated as a point estimate into this analysis [Exponent 2001; Ruby et al. 2002 (submitted)]. This study simulated human gastrointestinal conditions to determine the bioaccessible fraction of PCDD/Fs in Midland soils. The results indicate that, on average, only 25% of PCDD/Fs will dissolve into the gastrointestinal lumen and be available for systemic absorption. A distribution of site-specific soil criteria indicates that a concentration of 1.5 ppb would pose no greater than a 1 in 100,000 risk at the 95th percentile level of exposure for residents in Midland, Michigan while 4 ppb would be protective at the 50th percentile level.

LB30 TCDD DECREASES ESTRADIOL PRODUCTION BY ALTERING THE ENZYME EXPRESSION AND ACTIVITY OF 17 α -HYDROXYLASE/17, 20-LYASE CYTOCHROME P450 (P450C17) OF HUMAN LUTEINIZED GRANULOSA CELLS (HLGC).

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TCDD decreases estradiol (E2) production by hLGC without altering either E2 metabolism or cytochrome P450 aromatase activity. In the present study hLGC were used to analyze the fate of different 85 substrates of P450c17 in the presence or absence of TCDD. hLGC were plated on plastic culture dishes in medium with 2 IU/ml of hCG. TCDD (10 nM) or vehicle only were added directly onto the dishes at the time of medium change every 48 hours for 8 days to test the hypothesis that exogenous substrate of P450c17 would reduce the TCDD effects on E2 synthesis. In addition, using a recently developed antibody for the human P450c17 and an activity assay for 17,20-lyase we tested the hypothesis that TCDD targets P450c17 activity. When hLGC were treated with TCDD in the presence of various doses of exogenous DHEA there was an exponential and dose related increase in the E2 production and the TCDD effect on lowering the E2 disappeared. In contrast, when hLGC were incubated with 0-10 μ M of pregnenolone or 17 β -hydroxy-pregnenolone no change in E2 production was observed and this treatment did not modify the effect of TCDD on E2 production. Western immunoblot analysis demonstrated that TCDD treatment decreased the expression of P450c17 by 50% ($P < 0.05$) and 17,20-lyase activity of this protein by 65% ($P < 0.05$). We conclude that one of the rate limiting steps in estrogen synthesis by hLGC is the 17,20-lyase activity of P450c17 and the inhibition of E2 secretion by TCDD involves steps related to the production of androgens, i.e. the 17,20-lyase activity of P450c17 and not earlier steps such as hydroxylation of Pe. Thus, these data identify the specific molecular target for TCDD toxicity in hLGC as P450c17 expression and the 17,20-lyase activity of this enzyme complex. Supported by: NIEHS.

LB31 COMPARATIVE ARYL HYDROCARBON RECEPTOR-HORMONE RECEPTOR CROSSTALK IN BREAST AND PROSTATE CANCER CELLS.

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2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is a ligand for the aryl hydrocarbon receptor (AhR) and inhibits growth of both breast (T47D) and prostate (LNCaP

and 22RV1) cancer cells in culture. One mechanism for TCDD-induced inhibitory activity in breast cancer cells involves activation of proteasome-dependent degradation of estrogen receptor α (ER α). This study investigated the comparative effects of TCDD and steroid hormones on receptor proteins in breast and prostate cancer cells. T47D, LNCaP and 22RV1 cells express the androgen receptor (AR) and, after treatment with 10 nM dihydrotestosterone (DHT) for 24 h, there was a significant 4- to 6-fold upregulation of immunoreactive AR protein, and this increase was observed within 1 to 3 h after treatment in all three cell lines. In contrast, 17 β -estradiol (E2) decreased AR levels in T47D cells, whereas in prostate cancer cells, AR levels were slightly elevated by E2. TCDD induces proteasome-dependent degradation of ER α in breast cancer cells; however, after treatment with 10 nM TCDD for up to 24 h, only minimal changes in immunoreactive AR protein were observed in T47D breast and 22RV1 prostate cells. Combined treatment with DHT plus TCDD resulted in some decreases in AR protein in 22RV1 cells (compared to treatment with DHT alone), whereas TCDD did not alter upregulation of AR protein by DHT in T47D cells. Thus, the effects of TCDD on AR and other hormone receptors in prostate and breast cancer cells are dependent on cell context, and current studies are focused on the mechanisms of AhR-hormone receptor crosstalk in both breast and prostate cancer cell lines (Supported by DOD-USAMRMC 17-02-1-0147 and NIH ES09106).

LB32 NATURALLY OCCURRING AH-RECEPTOR AGONISTS IN THE DIET: WHY A VEGAN DIET WON'T REDUCE YOUR "DIOXIN DOSE."

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The presence of naturally occurring aryl hydrocarbon receptor (AhR) agonists in the human diet has implications for a biologically-based risk assessment of tetrachlorodibenzo-*p*-dioxin (TCDD), where AhR binding is the 'key event' and risks from numerous exogenous compounds are estimated on the basis of AhR agonism. This analysis compares the daily TCDD equivalency (TEQ) dose of a single dietary-derived AhR agonist to "background" dietary TCDD doses. The daily dose of indolo[3,2-*b*]carbazole (ICZ) was estimated based on the dietary intake of indole-3-carbinole (I3C); the dietary dose of I3C was derived from published estimates of vegetable consumption rates and the vegetable content of this compound. A TCDD-equivalency factor (TEF) for ICZ (0.01) was derived from *in vitro* and cell culture studies. A "background" daily dietary dose of 40 pg PCDD/F TEQ/day was assumed. The 30-year average daily intake, accumulated body burden, and area-under-the curve (AUC) TEQ doses were then calculated using published estimates of bioavailability and biological half-life for ICZ and TCDD. For each dose metric, the steady-state dose of ICZ was 73% or more of the total TEQ dose. This comparison, which evaluated only one of the hundreds of naturally occurring dietary AhR agonists, suggests that reducing the intake of animal products might not achieve any significant reduction in the overall human intake of AhR agonists. Additional research on the indoles and several other naturally occurring "dioxins" is needed to properly characterize their AhR agonist and antagonist effects.

LB33 AhRC-PCR ASSAY QUANTIFIES AhR ACTIVATION BY REAL-TIME PCR.

J Willey, Hybrizyme Corporation, Raleigh, NC. Sponsor: S Dertinger.

The aryl-hydrocarbon receptor (AhR) mediates most, if not all, of the toxicity related to dioxin and dioxin-like compounds. Activation of the AhR to a DNA-binding form by dioxin-like compounds was demonstrated and quantified using a novel format. When activated, the AhR binds ARNT (AhR nuclear translocator) protein to form a DNA-binding complex. The specific DNA element bound is termed the dioxin-responsive element (DRE). In our format, the AhR/ARNT/DRE complex is captured in a microwell. Unbound components are washed away and the bound DRE is PCR-amplified and quantified using real-time detection technologies. The DNA signal correlates to the concentration of 2,3,7,8-tetrachloro-*p*-dioxin or beta-naphthoflavone present in the standard. The assay has a linear performance over 2 orders of magnitude and low ppt (pg/ml) sensitivity. This method will prove useful for environmental and food monitoring,



Aryl hydrocarbon receptor-mediated inhibition of LNCaP prostate cancer cell growth and hormone-induced transactivation

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Abstract

LNCaP prostate cancer cells express the aryl hydrocarbon receptor (AhR), and treatment with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) induces CYP1A1 protein and an Ah-responsive reporter gene. Similar results were obtained with the selective AhR modulator 6-methyl-1,3,8-trichlorodibenzofuran (6-MCDF); however, TCDD but not 6-MCDF induced degradation of the AhR protein. TCDD and 6-MCDF inhibited growth of LNCaP cells, and inhibitory AhR-androgen receptor (AR) crosstalk was investigated in cells transfected with constructs containing the androgen-responsive probasin promoter (−288 to +28) (pPB) or three copies of the −244 to −96 region of this promoter (pARR₃). Ten nanomolar dihydrotestosterone (DHT) and 17β-estradiol (E2) induced transactivation in LNCaP cells transfected with pPB or pARR₃; however, inhibitory AhR-AR crosstalk was observed only with the latter construct. 6-MCDF and TCDD did not inhibit DHT- or E2-induced transactivation in ZR-75 human breast cancer cells, indicating that these interactions were promoter and cell context-dependent. Both E2 and DHT stabilized AR protein in LNCaP cells; however, cotreatment with TCDD or 6-MCDF decreased AR protein levels. These results indicate that inhibitory AhR-AR crosstalk in prostate cancer cells is complex and for some responses, AR protein stability may play a role.

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Keywords: Ah receptor; Androgen receptor; Inhibitory crosstalk; LNCaP cells

1. Introduction

Prostate cancer is the most commonly diagnosed cancer in North American men and it is estimated that there are over 300,000 newly diagnosed cases each year [1,2]. The incidence and mortality rates from prostate cancer are increasing and this is due, in part, to an increasingly aging population and the higher incidence of this disease in older men [3,4]. Prostate cancer therapy is dependent on the stage of the tumor and androgen receptor (AR) expression. Early stage androgen-responsive prostate cancers can be treated by castration or with antiandrogens or drugs that block androgen-induced responses including steroidal antiandrogens (cyproterone), luteinizing hormone releasing hormone (LHRH) analogs, nonsteroidal antiandrogens (flutamide, nilutamide, bicalutamide), and the potent estrogenic drug diethylstilbestrol (reviewed in [5–8]). In addition, there are several novel strategies for treatment of prostate cancer and other tumor-types and these include targeting of critical

genes involved in tumor cell growth and metastasis (e.g., antiangiogenic drugs, antisense therapy) [9–13]. Ligands for nuclear receptors (NR) are also being developed for treatment of prostate cancer through inhibitory NR-AR crosstalk that involves various compounds that bind the retinoid acid/X-receptors (retinoids), vitamin D receptor (calcitriol), and peroxisome proliferator activate receptor γ (thiazolidinedione-derived drugs) [14–26]. A recent study in androgen-responsive LNCaP prostate cancer cells showed that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a ligand for the aryl hydrocarbon receptor (AhR), inhibited testosterone-induced cell proliferation and gene/reporter gene expression [27].

The AhR was initially identified as the intracellular receptor that bound TCDD and related toxic halogenated aromatic hydrocarbons [28,29]; however, more recent studies show that chemoprotective phytochemicals and other structurally-diverse chemicals also interact with this receptor [30]. There is also evidence that the AhR is a potential target for drug development since long-term feeding studies with TCDD in female Sprague–Dawley rats showed that development of several age-dependent cancers including

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17 β -estradiol (E2)-dependent mammary and uterine tumors were inhibited [31]. Subsequent studies have demonstrated inhibitory AhR-ER crosstalk in the rodent uterus, rodent mammary tumors, breast and endometrial cancer cells [28,32–34]. In addition to the reported growth inhibitory effects of TCDD in prostate cancer cells, recent studies show that AhR agonists also inhibit growth of pancreatic cancer cells [35].

Research in this laboratory is focused on development of selective AhR modulators (SAhRMs) that exhibit tissue-specific AhR agonist or antagonist activity [36,37]. Alternate substituted (1,3,6,8- or 2,4,6,8-) alkyl polychlorinated dibenzofurans, typified by 6-methyl-1,3,8-trichlorodibenzofuran (6-MCDF), are relatively non-toxic and inhibit prototypical AhR-mediated toxic responses in rodent models (i.e., AhR antagonists) but exhibit selective AhR-dependent antiestrogenic and antitumorigenic activities in mammary tumor models [38–46]. 6-MCDF also inhibits growth of some ER-negative breast cancer [47] and pancreatic cancer cells [35]. This paper describes inhibition of LNCaP prostate cancer cell growth by TCDD and 6-MCDF, and both compounds also inhibit E2- and androgen-induced transactivation in LNCaP cells transfected with an androgen-responsive construct containing probasin gene promoter inserts.

2. Materials and methods

2.1. Chemicals, biochemicals, and plasmids

Fetal bovine serum (FBS) was obtained from Summit Biotechnology (Fort Collins, CO). RPMI 1640 medium, phenol-free Dulbecco's modified Eagle's medium/F-12 medium, phosphate-buffered saline, 100 $\times$ antibiotic/antimycotic solution, *N*-[2-hydroxyethyl]piperazine-*N'*[2-ethanesulfonic acid] (HEPES), 17 β -estradiol (E2), and dihydrotestosterone (DHT) were purchased from Sigma; 5 $\times$ reporter lysis buffer and luciferin were purchased from Promega (Madison, WI). Reagents for β -galactosidase analysis were purchased from Applied Biosystems (Foster City, CA). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and 6-methyl-1,3,8-trichlorodibenzofuran (6-MCDF) were synthesized in this laboratory. Forty percent polyacrylamide was obtained from National Diagnostics (Atlanta, GA). PB-luc and ARR₃TK-luc [48] constructs were the generous gifts of Dr. Robert J. Matusik (Vanderbilt University Medical Center, Nashville, TN). Human AR (hAR) expression plasmid [49] was kindly provided jointly by Drs. Kerry L. Burnstein (University of Miami School of Medicine) and Michael J. McPhaul (U.T. Southwestern Medical School, Dallas, TX). The pcDNA3.1- β -gal plasmid was obtained from Invitrogen (Carlsbad, CA). The pDRE₃-luciferase reporter plasmid was constructed in this laboratory and contains three tandem consensus dioxin response elements (DRE) (TCT TCT CAC GCA ACT CCG A—a single DRE sequence). All other chemicals and biochemicals were the

highest quality available from commercial sources. Scheduled substances were procured, stored, and disposed in compliance with relevant federal and state laws.

2.2. Transient transfection assays

ZR-75 human breast cancer and LNCaP human prostate cancer cells were obtained from American Type Culture Collection (Manassas, VA) and were maintained in RPMI 1640 medium supplemented with 10% FBS, 1% antibiotic/antimycotic solution, 1.5 g/l sodium bicarbonate, and 10 mM HEPES, final pH of 7.4. Cells were seeded at 2.75×10^5 per 22-mm well in DME-F12 without phenol red, supplemented with 2.5% charcoal-stripped FBS. After 24 h, cells were transfected using Lipofectamine and Plus reagents (Invitrogen) according to manufacturer's instructions. LNCaP and ZR-75 cells were transfected with 500 ng per well of either reporter plasmid, and 250 ng per well of pcDNA3.1- β -gal (Invitrogen) as the internal control. In addition, ZR-75 cells were transfected with 500 ng hAR. Twenty-four hours after treatment, cells were harvested by scraping with 200 μ l per well of reporter lysis buffer. Lysates were centrifuged at $40,000 \times g$ and luciferase and β -galactosidase activity was assayed with 30 μ l of the supernatant extract per sample using a Lumicount luminometer (Perkin-Elmer, Boston, MA). Luciferase activity was normalized to β -galactosidase activity for each transfection well. Results of transfection experiments are expressed as means $\pm$ S.E. compared to the DMSO control group, which is set at 1.

2.3. Cell proliferation assay

After trypsinization and low-speed centrifugation, LNCaP cells were resuspended and counted using a Coulter cell counter (Beckman Coulter, Fullerton, CA). Cells were seeded at a density of 5×10^4 /35-mm well using DME-F12 without phenol red, supplemented with 2.5% charcoal-stripped FBS. Twenty-four hours after seeding, initial treatment was applied and then subsequently reapplied with fresh medium every two days until harvesting by trypsinization. Cells were counted after harvesting using a Coulter counter.

2.4. Fluorescence activated cell sorting analysis

Cells were analyzed on a FACS Calibur (Becton Dickinson, San Jose, CA) flow cytometer, equipped with a 15 mW air-cooled argon laser, using CellQuest (Becton Dickinson) acquisition software. Propidium iodide (PI) fluorescence was collected through a 585/42-nm bandpass filter, and list mode data were acquired on a minimum of 12,000 single cells defined by a dot plot of PI-width versus PI-area. Data analysis was performed in ModFit LT (Verity Software House, Topsham, ME) using PI-width versus PI-area to exclude cell

aggregates. FlowJo (Treestar, Inc., Palo Alto, CA) was used to generate plots summarized in Table 1.

2.5. Western immunoblot analysis

Cells were harvested 6 h after treatment using 200 μ l/22-mm well of ice cold lysis buffer (50 mM HEPES, pH 7.5, 500 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Triton-X 100, 1.5 mM $MgCl_2$, 1 mM EGTA) [46]. Lysates were centrifuged at $40,000 \times g$, and supernatant extract was collected. Whole cell extracts (50 μ g per sample) were separated by electrophoresis on a tiered 7.5% (top)/12.5% (bottom) SDS–polyacrylamide gel and transferred to PVDF membrane (Bio-Rad, Richmond, CA). The membrane was blocked with 5% milk (m/v) in tris-buffered saline 0.05% Tween (TBST). Membranes were incubated with primary antibodies for AR (sc-7305), cyclin D1 (sc-718), or p27 (sc-528) (each from Santa Cruz Biotechnology, Santa Cruz, CA) at 1:1000 in 5% milk/TBST for 3 h. Membranes were washed twice in TBST. Horseradish peroxidase (HRP)-conjugated secondary antibodies were applied at 1:5000 in 5% milk/TBST for 1 h. After two TBST washes, PVDF-bound antibodies were detected using a chemiluminescence kit (Western Lightning, Perkin-Elmer), ImageTek-H film (American X-Ray and Medical Supply, Rancho Cordova, CA) and an autoprocesor (Hope Macro-Med, Warminster, PA). Quantitation of the Western blot was performed using a Sharp JX-330 scanner (Sharp, Mahwah, NJ) and Zero-D software (Scanalytics, Billerica, MA). The experimental protocol used for Western blot analysis of CYP1A1, AhR, cyclin D1, p27 and Arnt protein were essentially as described above [46] using CYP1A1, AhR and Arnt antibodies purchased from Santa Cruz Biotechnology. In this experiment, cells were treated with 10 nM TCDD, 2 or 5 μ M 6-MCDF for 6 or 12 h. Results for quantitative comparisons of AR protein levels are expressed as means $\pm$ S.E. for three separate experiments, and levels were compared to the DMSO control group, which was set at 1.

3. Results

3.1. Effects of TCDD and 6-MCDF on AhR activation and growth of LNCaP cells

Previous studies reported that the AhR and Arnt mRNA are expressed in LNCaP cells and Ah-responsiveness was confirmed by induction of CYP1A1 mRNA and CYP1A1-dependent EROD activity by TCDD [27]. Results illustrated in Fig. 1A show that 10 nM TCDD, 2 and 5 μ M 6-MCDF induce CYP1A1 protein in LNCaP cells, and this is consistent with previous reports showing that 10 nM TCDD induces CYP1A1-dependent EROD activity [27]. Western blot analysis also confirmed expression of both AhR and Arnt proteins, and treatment with TCDD but

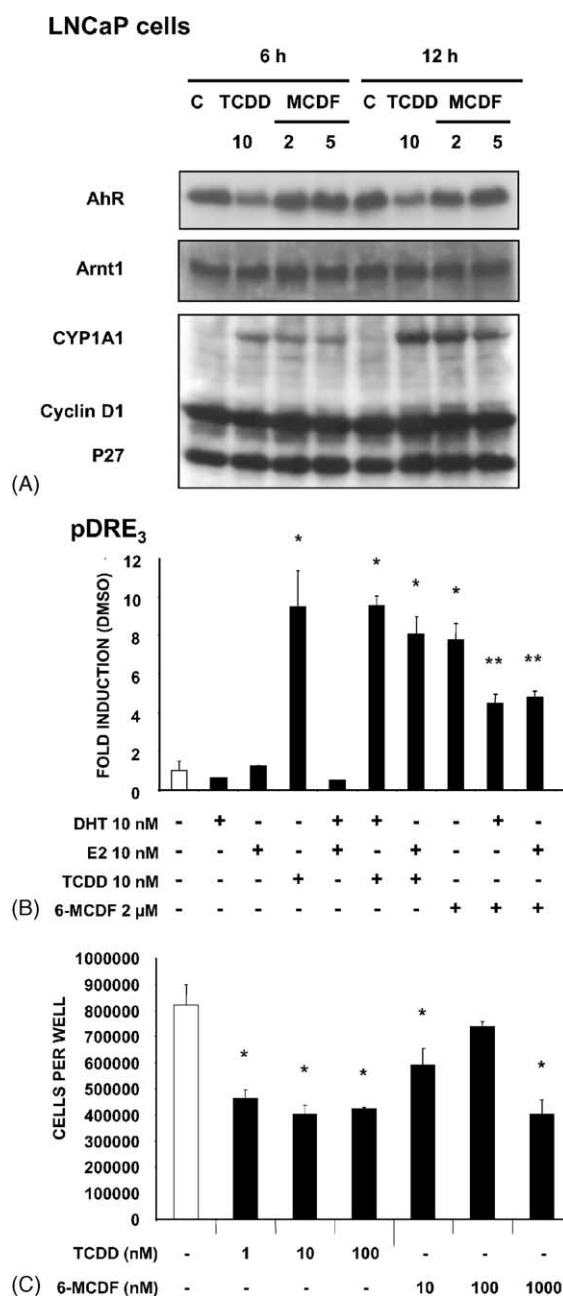


Fig. 1. Ligand-dependent AhR activation and growth inhibition in LNCaP cells. (A) Induction of CYP1A1 protein. LNCaP cells were treated with DMSO (C), 10 nM TCDD, 2 or 5 μ M 6-MCDF for 6 or 12 h, and whole cell lysates were analyzed by Western blot analysis as described in Section 2. Antibodies were used to detect the AhR, Arnt, CYP1A1, cyclin D1 and p27 proteins. (B) Activation of pDRE₃. LNCaP cells were transfected with pDRE₃, treated with various compounds and luciferase activity was determined as described in Section 2. Significant induction ($P < 0.05$) is indicated with an asterisk and inhibition of TCDD- or 6-MCDF-induced activity is also indicated (**). (C) Inhibition of LNCaP cell growth by TCDD and 6-MCDF. Cells were cultured for six days, treated with different concentrations of TCDD or 6-MCDF, and cell numbers were determined as described in Section 2. Significant ($P < 0.05$) growth inhibition is indicated by an asterisk. All results are presented as means $\pm$ S.E. for three replicate determinations for each treatment group. Growth inhibition in some of the groups was observed after 2 to 4 days.

Table 1

Effects of TCDD on cell cycle progression in LNCaP prostate cancer cells^a

Treatment	Percent distribution		
	G ₀ /G ₁	G ₂ /M	S
DMSO	70.300 ± 1.779	10.973 ± 0.544	18.7 ± 1.258
TCDD (10 ⁻⁹ M)	74.300 ± 0.751*	10.633 ± 0.376	14.7 ± 0.520*
TCDD (10 ⁻⁸ M)	75.367 ± 0.636*	10.300 ± 0.153	14.3 ± 0.666*
TCDD (10 ⁻⁷ M)	77.500 ± 0.451*	8.943 ± 0.471	13.567 ± 0.176*
DHT (10 ⁻⁸ M)	73.400 ± 1.179	9.433 ± 1.011	17.167 ± 0.296

^a LNCaP cells were treated as indicated for 48 h and the percentage distribution of cells in G₀/G₁, G₂/M, and S phases were determined by FACS analysis as described in Section 2. Significant ($P < 0.05$) effect compared to DMSO are indicated by an asterisk.

not 6-MCDF decreased expression of the AhR. Expression of other proteins including Sp1, cyclin D1 and p27 were unaffected by the treatments and serve as loading controls. Results illustrated in Fig. 1B also show that treatment of LNCaP cells with 10 nM TCDD induced luciferase activity >9-fold compared to solvent control (DMSO) in cells transfected with pDRE₃. In contrast, 10 nM DHT, 10 nM E2 and E2 plus DHT did not significantly induce activity, and neither DHT or E2 in combination with TCDD affected induced activity. 6-MCDF (2 μM), a prototypical SAhRM, also induced luciferase activity (>7-fold), and this was consistent with the induction of CYP1A1 by 6-MCDF. 6-MCDF is a much less potent agonist for activation of CYP1A1 or DRE-dependent activities in breast cancer cells [42]. Both E2 and DHT in combination with 6-MCDF significantly inhibited 6-MCDF-induced activity, whereas in cells treated with TCDD in combination with E2 or DHT, inhibitory interactions were not observed.

The comparative effects of TCDD and 6-MCDF on growth of LNCaP cells were also determined in cells treated with solvent control and different concentrations of the AhR agonists for 6 days. The results show that TCDD (1–100 nM) significantly inhibited proliferation of LNCaP cells, and growth inhibition was also observed for 6-MCDF (Fig. 1B). Both compounds inhibited ≥50% cell growth at one or more concentrations. Similar experiments were also carried out with 6-MCDF and TCDD in LNCaP cells also treated with different concentrations of DHT (up to 10 nM). Hormone-induced cell growth was not observed; however, both 6-MCDF and TCDD inhibited growth of LNCaP cells in the presence of DHT (data not shown). These results confirm that LNCaP cells are Ah-responsive and both TCDD and 6-MCDF inhibit LNCaP cell proliferation. The effects of TCDD on cell cycle progression was also determined in LNCaP cells treated with 1.0, 10 and 100 nM TCDD for 48 h followed by FACS analysis (Table 1). The results show that TCDD induced a small but significant increase in the percentage of cells in G₀/G₁ and a decrease of cells in S phase, whereas solvent (DMSO) and DHT (10 nM) exhibited minimal differences.

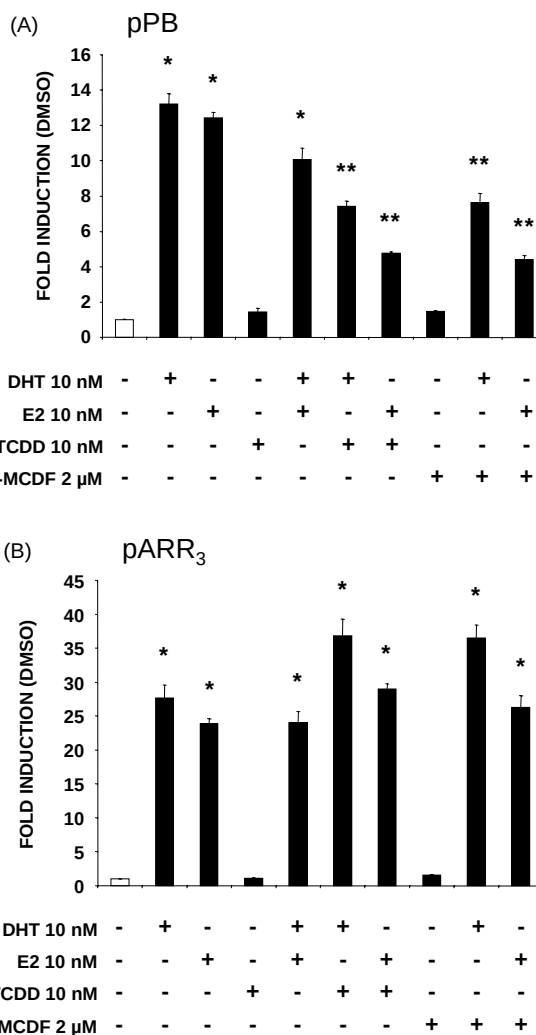


Fig. 2. Inhibition of AR-dependent transactivation by TCDD and 6-MCDF. LNCaP cells were transfected with pPB (A) or pARR₃ (B), treated with hormone or AhR agonist alone or in combination, and luciferase activity was determined as outlined in Section 2. Significant ($P < 0.05$) induction by compounds alone is indicated by an asterisk, and significant ($0 < 0.05$) inhibitory effects observed in cotreatment studies are also indicated (**). Results are expressed as means ± S.E. for three replicate determinations for each treatment group.

3.2. Inhibitory AhR-AR crosstalk in LNCaP cells transfected with androgen-responsive constructs

Jana et al. [27] previously reported that TCDD inhibited testosterone-induced luciferase activity in LNCaP cells transfected with an androgen-responsive construct containing the mouse mammary tumor virus (MMTV) promoter. Inhibition of testosterone-induced PSA protein or mRNA by 100 nM TCDD was reported but not quantitated, and the magnitude of inhibition was minimal. Therefore, we further investigated inhibitory AhR-AR crosstalk in LNCaP cells transfected with pPB which contains the −286 to +28 region of the androgen-responsive probasin gene promoter (Fig. 2A). There was a >13-fold increase in luciferase

activity in LNCaP cells treated with 10 nM DHT and transfected with pPB and the induced response was significantly inhibited after cotreatment with DHT plus TCDD. Similar inhibitory responses were also observed using 2 μ M MCDF (Fig. 2A), whereas TCDD and MCDF alone did not significantly induce activity. Surprisingly, 10 nM E2 alone induces luciferase activity in LNCaP cells transfected with pPB, and the hormone-induced response is significantly decreased in cells cotreated with E2 plus TCDD or 6-MCDF (Fig. 2A).

The pARR₃ construct contains three tandem (3) copies of the probasin androgen response element, and was used to further investigate inhibitory AhR-AR crosstalk and the androgenic activity of E2. Ten nanomolar DHT induced a >27-fold increase in luciferase in LNCaP cells transfected with pARR₃; however, for this construct, cotreatment with DHT plus MCDF or TCDD did not decrease DHT-induced activity (Fig. 2B). E2 (10 nM) also induced luciferase activity (>24-fold) in cells transfected with pARR₃; however, in cells cotreated with E2 plus TCDD or MCDF, activity was not significantly decreased compared to that observed for E2 alone. These results confirmed that both DHT and E2 activated gene expression in cells transfected pPB or pARR₃; however, inhibitory effects of AhR agonists were observed only for the former construct.

The unexpectedly high AR agonist activity of E2 compared to DHT in LNCaP cells were further investigated in cells transfected with pPB and treated with hormones and antiandrogens or antiestrogens. Induction of luciferase activity by 10 nM DHT and E2 in LNCaP cells transfected with pPB was inhibited in cells cotreated with the hormone plus 10 μ M HPTE, an AR antagonist (Fig. 3A). However, in parallel studies, the “pure” antiestrogen ICI 182780 also significantly inhibited E2-induced activity, whereas only minimal inhibition was observed in LNCaP cells treated with DHT plus ICI 182780. In a parallel experiment in LNCaP cells transfected with pARR₃, both HPTE and ICI 182780 inhibited DHT and E2-induced luciferase activity (Fig. 3B), whereas 1 μ M flutamide, an AR antagonist, caused only minimal decreases in hormone-induced activity (Fig. 3C). HPTE is also an ER α agonist and ER β antagonist [50] and the results obtained for both HPTE and ICI 182780 suggest a possible role for ER β in mediating activation of pPB and pARR₃. However, previous studies show that endogenous ER β is insufficient for E2-induced transactivation in LNCaP cells transfected with pERE₃, a construct containing three tandem estrogen responsive elements (ERE₃) [51,52], suggesting that activation of pPB or pARR₃ is ER β -independent. Therefore, in order to confirm the role of AR in mediating these responses, we further investigated hormone activation of pPB and inhibitory AhR-AR crosstalk in ZR-75 cells which express minimal AR protein [53]. Results in Fig. 4A show that DHT, E2, TCDD and MCDF do not activate reporter gene activity in ZR-75 cells transfected with pPB alone; however, both DHT and E2 induced luciferase activity in cells cotransfected with pPB and hAR expression plasmid (Fig. 4B). Induction by E2 was significant but

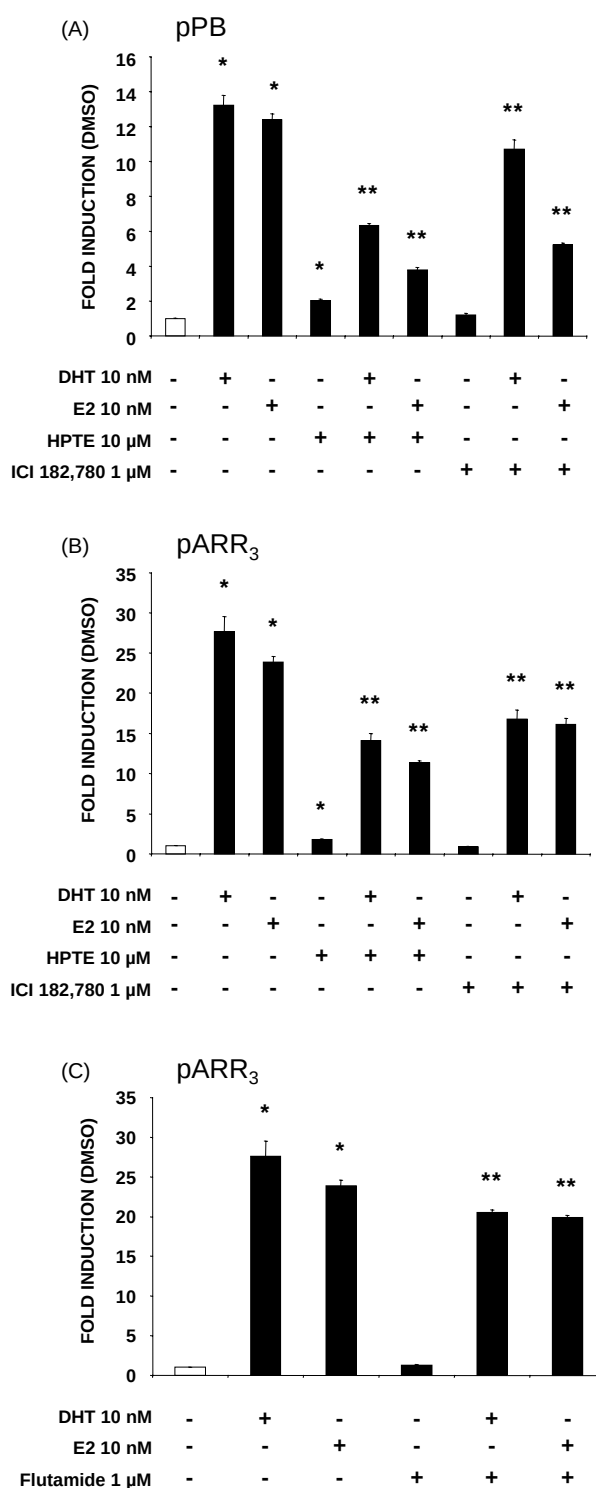


Fig. 3. Inhibition of AR-dependent transactivation by antiandrogens and antiestrogens in LNCaP cells. Cells were transfected with pPB (A), pARR₃ (B) or pPB (C), treated with various compounds, and luciferase activity was determined as described in Section 2. Significant ($P < 0.05$) induction by compounds alone is indicated by an asterisk, and significant ($P < 0.05$) inhibitory effects observed in cotreatment studies is also indicated (**). Results are expressed as means $\pm$ S.E. for three replicate determinations for each treatment group.

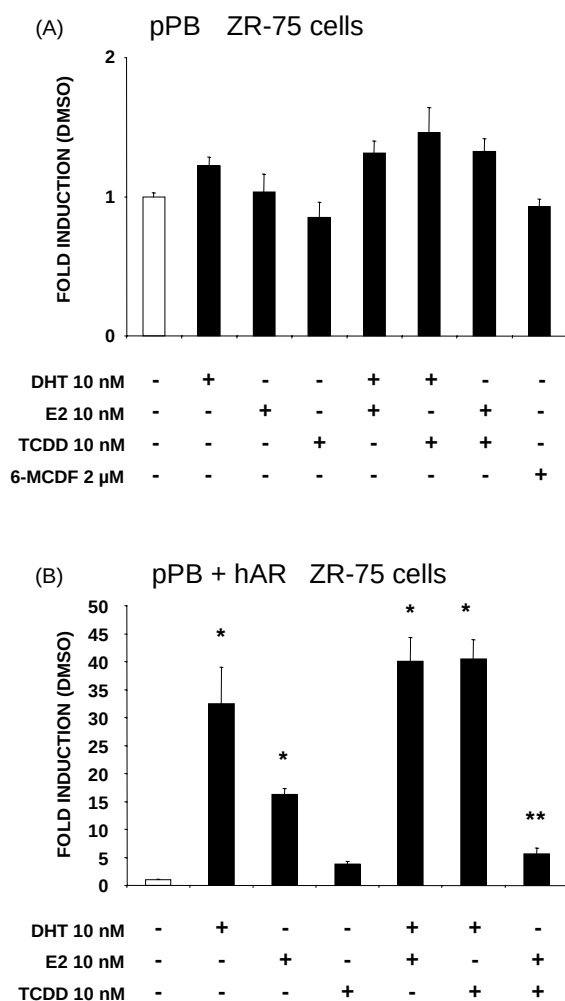


Fig. 4. Inhibition of hormone-induced transactivation in ZR-75 breast cancer cells transfected with pPB. (A) Transfection with pPB alone. ZR-75 cells were transfected with pPB, treated with various compounds and luciferase activity was determined as described in the Materials and Methods. No significant induction was observed in any of the treatment groups. (B) Transfection with pPB and hAR. Cells were transfected and treated as described in (A) except that 500 ng of hAR expression plasmid was also transfected. Significant ($P < 0.05$) induction by compounds alone is indicated by an asterisk and significant inhibitory effects observed in cotreatment studies is also indicated (**). Results are expressed as means $\pm$ S.E. for three replicate determinations for each treatment group.

lower than observed for DHT in ZR-75 cells, and TCDD inhibited E2 but not DHT-induced activity in cells cotreated with hormone plus TCDD. Similar results were observed in duplicate experiments confirming that E2-dependent transactivation of pPB was AR-dependent. However, it was also evident that there were important differences between the interaction of TCDD and DHT in LNCaP and ZR-75 cells since TCDD did not inhibit DHT-induced luciferase activity in the latter cell line. This suggests that inhibitory AhR-AR crosstalk is cell context-dependent for the pPB promoter.

3.3. Effects of various treatments on AR, cyclin D1 and p27 protein levels in LNCaP cells

Levels of AR protein expression may influence androgen-responsiveness and inhibitory AhR-AR crosstalk, and the results in Fig. 5A demonstrate levels of immunoreactive AR protein in LNCaP cells after various treatments. Preliminary studies in LNCaP and other cell lines indicated that any changes in AR expression were observed within 6–12 h after treatment (data not shown) and a 6 h time point was selected for this study. Treatment with 10 nM DHT, 10 nM E2 or DHT plus E2 resulted in a significant increase in AR levels. In contrast, 10 nM TCDD and 2 μ M 6-MCDF alone did not significantly affect levels of AR protein; however, in combination with DHT, there was a significant decrease in AR levels compared to cells treated with DHT alone. TCDD in combination with E2 also decreased AR levels compared to those observed in cells treated with E2 alone. In contrast, levels of immunoreactive p27 protein were not significantly changed by any of the treatments (also observed in studies summarized in Fig. 1A), and served as a loading control for this experiment. In a separate study, the effects of the antiandrogen HPTE and the antiestrogen ICI 182780 alone and in combination with E2 or DHT on AR levels were also determined (Fig. 5B). Ten micromolar HPTE alone did not affect AR levels in LNCaP cells, whereas ICI 182780 treatment increased AR levels compared to DMSO (solvent) treatment. Hormone (E2 or DHT)-induced upregulation of AR protein was not decreased cotreatment with HPTE or ICI 182780. Cyclin D1 protein was not significantly changed in this study and served as a loading control (also see Fig. 1A). These data demonstrate that various treatments differentially modulate AR protein levels in LNCaP cells, and current studies are focused on the influence of ligand-induced changes in AR expression and the magnitude of hormone-induced transactivation.

4. Discussion

The AhR was initially characterized by its high affinity, low capacity binding to TCDD and related toxic halogenated aromatic hydrocarbons [54]. However, recent studies have demonstrated that the AhR also interacts with structurally diverse synthetic chemicals, drugs, endogenous biochemicals, and phytochemicals [30,55–57]. Moreover, many of these compounds such as synthetic retinoids, bioflavonoids, indole-3-carbinol and diindolylmethane (DIM) exhibit chemoprotective and anticarcinogenic properties in laboratory animal studies [58–63]. 6-MCDF is an example of a relatively non-toxic synthetic AhR agonist/antagonist that inhibits several TCDD-induced toxic responses including cleft palate, immunotoxicity and porphyria in mice and CYP1A1 in both in vivo and in vitro models [38–41]. However, 6-MCDF exhibits selective AhR agonist activity as an antiestrogen and inhibits E2-dependent mammary tumor

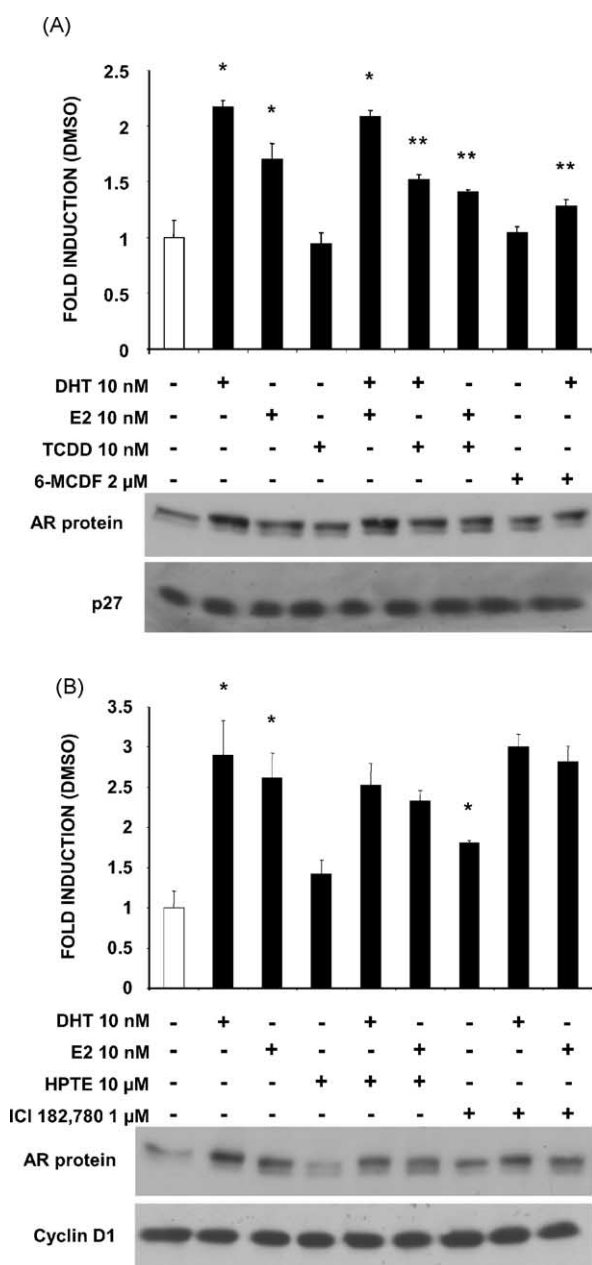


Fig. 5. AR protein expression in LNCaP cells treated with hormones, AhR agonists, antiandrogens and antiestrogens. (A) AR protein expression in cells treated with hormones and AhR agonists. LNCaP cells were treated with DHT, E2, TCDD, 6-MCDF and their combinations for 6 h, and AR protein levels in whole cell lysates were determined by Western blot analysis as described in Section 2. p27 protein was also determined for this experiment; p27 was essentially unchanged in all of the treatment groups and serves as a loading control for this experiment. (B) AR protein expression in cells treated with hormones, antiandrogens and antiestrogens. AR protein levels were determined essentially as described in (A) and blots were stripped and reprobed with cyclin D1 antibodies. Cyclin D1 protein was unchanged in this experiment and serves as a loading control. For studies illustrated in (A) and (B), significant ($P < 0.05$) increases in AR protein levels by individual compounds are indicated by an asterisk, and significant ($P < 0.05$) decreases in the cotreatment groups are also indicated (**). Results are expressed as means $\pm$ S.E. for three replicate determinations for each treatment group.

growth (in vivo) and breast/endometrial cancer cell growth [42–46].

Recent studies show that 6-MCDF also inhibits E2-independent pancreatic cancer cell growth [35], and results of this study show that both TCDD and 6-MCDF inhibit growth of LNCaP cells (Fig. 1C), decrease the percentage of cells in S phase, and increase the percentage in G_0/G_1 (Table 1). Although the percentage of cells in G_0/G_1 and S phase are significantly affected by TCDD, the changes are relatively small suggesting that modulation of cell cycle genes by TCDD may not be a critical pathway for growth inhibition. Treatment of LNCaP cells with up to 10 nM DHT did not increase cell growth (data not shown) or $G_0/G_1 \rightarrow$ S phase progression (Table 1); however, TCDD and 6-MCDF also inhibited LNCaP cell growth in the presence of DHT (data not shown). In addition, 6-MCDF and TCDD did not affect expression of cyclin D1 or p27 (Figs. 1A and 5), and only minimal expression of p21 was observed in the treatment groups (data not shown). Current studies are further investigating the mechanisms of LNCaP cell growth inhibition by AhR agonists.

Jana and coworkers [27,64] have reported inhibitory AhR-AR crosstalk in LNCaP cells and showed that 10 or 100 nM testosterone inhibited EROD activity induced by 100 nM TCDD and that TCDD inhibited testosterone-induced activation of an androgen-responsive construct containing the MMTV promoter. Results in Fig. 1A and B confirm the Ah-responsiveness of LNCaP cells. Both the AhR and Arnt proteins are expressed in LNCaP cells, and CYP1A1 protein is induced by TCDD and 6-MCDF. The induction of CYP1A1 by 6-MCDF was surprising since previous studies in breast cancer cells, rodent mammary tumors, and rodent liver show that this compound only weakly induces CYP1A1, and in cotreatment studies (TCDD + 6-MCDF), 6-MCDF inhibits induction of CYP1A1 by TCDD [38–45]. Treatment of LNCaP cells with TCDD resulted in decreased AhR protein expression, and this is consistent with studies in other cell lines where TCDD activates proteasome-dependent degradation of the AhR [45,65–67]. In contrast, 2 or 5 μM 6-MCDF did not decrease AhR protein levels, and differences between the effects of TCDD and 6-MCDF correlated with reports showing that interactions of these compounds with the AhR induce different conformation of the bound receptor complex [68]. Thus, although TCDD and 6-MCDF induce similar responses, there are differences in their mode of action. TCDD and 6-MCDF also induced luciferase activity in cells transfected with pDRE3 (Fig. 1B). Hormone-dependent decreases in TCDD-induced activity were not observed, whereas both E2 and DHT inhibited luciferase activity induced by 6-MCDF. This is consistent with a potential squelching mechanism where the AR and AhR compete for common cofactors, and inhibitory AR-AhR crosstalk is observed only with a less potent AhR agonists.

Inhibitory AhR-AR crosstalk was investigated using two related androgen-responsive constructs, pPB and pARR3. pPB contains the –286 to +28 region of the probasin gene

promoter and the more androgen-responsive pARR₃ construct contains three copies of the –244 to –96 region of the rat probasin gene promoter [48,69]. The results in Fig. 2 demonstrate that both E2 and DHT induce luciferase activity in LNCaP cells transfected pPB and pARR₃. Significant inhibition of DHT- and E2-induced activity by 10 nM TCDD or 6-MCDF was observed in cells transfected with pPB but not pARR₃. The inhibitory AhR-AR crosstalk in LNCaP cells transfected with pPB complements results of previous studies using a construct with a human PSA gene promoter insert [27]. The results obtained for pARR₃ and pPB also demonstrate that inhibitory crosstalk is promoter specific; differences may be due to promoter flanking sequences within the PB promoter that are not present in the pARR₃ construct and this is currently being investigated.

Both E2 and DHT activated pPB and pARR₃ in LNCaP cells, and patterns of inhibition by antiandrogens and antiestrogens were comparable (Figs. 2 and 3). Moreover, activation of pPB in AR-negative AR-75 cells [70] required cotransfection with AR expression plasmid (Fig. 4). These data are consistent with previous results showing that the mutant AR (Thr877Ala) expressed in LNCaP cells exhibits increased responsiveness to E2 [71,72].

Studies in this laboratory have demonstrated that inhibitory AhR-ER α crosstalk is associated with proteasome-dependent downregulation of ER α that results in limiting levels of this receptor [70]. Moreover, a recent report also showed that inhibition of androgen-induced transactivation by genistein in LNCaP cells was associated with genistein-induced downregulation of the AR [71]. We therefore investigated ligand-dependent changes in AR protein levels in LNCaP and other prostate and breast cancer cells, and preliminary time-course studies showed that AR levels stabilized within 6–24 h after treatment with hormones and/or their inhibitors. Results in Fig. 5 illustrate ligand-dependent changes in AR protein levels after treatment with hormones, AhR agonists, antiandrogen/antiestrogen compounds and their combinations. DHT increased levels of AR in LNCaP cells as previously reported [73,74]; similar responses were observed for E2 and this parallels the androgen-like activity of E2 in transactivation assays (Figs. 2–4). HPTE and ICI 182780 alone also increased AR levels but did not affect hormone-induced upregulation of AR protein. HPTE interactions with AR differ from the AR antagonist bicalutamide which downregulates AR and prevents DHT-induced upregulation of AR in LNCaP cells [73]. AhR agonists also blocked hormone-induced upregulation of AR protein and this paralleled the inhibitory AhR-AR crosstalk observed in transfection studies with pPB (Fig. 2). This suggests that modulation of AR protein by the AhR may contribute to inhibitory AhR-AR interactions; however, other factors, including promoter context, are important.

In summary, results of this study demonstrate that TCDD and the SAHRM 6-MCDF inhibit growth of LNCaP prostate cancer cells and inhibit hormone-induced upregulation of

AR protein. In contrast to AhR-dependent downregulation of ER α in breast cancer cells, AhR agonists alone did not affect AR levels in LNCaP cells and inhibitory AhR-AR crosstalk in transactivation experiments was promoter-dependent. These results suggest that ligand-dependent interactions between the AhR and AR signaling pathways are complex and current studies are investigating which key growth regulatory genes in LNCaP cells are targeted by the AhR. (Supported by Department of the Army DAMD17-02-1-0147).

References

- [1] S.H. Landis, T. Murray, S. Bolden, P.A. Wingo, Cancer statistics, CA, Cancer J. Clin. 48 (1998) 6–29.
- [2] A. Shibata, J. Ma, A.S. Whittemore, Prostate cancer incidence and mortality in the United States and the United Kingdom, J. Natl. Cancer Inst. 90 (1998) 1230–1231.
- [3] X. Gao, A.T. Porter, D.J. Grignon, J.E. Pontes, K.V. Honn, Diagnostic and prognostic markers for human prostate cancer, Prostate 31 (1997) 264–281.
- [4] A. Chiarodo, National Cancer Institute roundtable on prostate cancer: future research directions, Cancer Res. 51 (1991) 2498–2505.
- [5] M.D. Sadar, M. Hussain, N. Bruchovsky, Prostate cancer: molecular biology of early progression to androgen independence, Endocr. Relat. Cancer 6 (1999) 487–502.
- [6] L. Klotz, Hormone therapy for patients with prostate carcinoma, Cancer 88 (2000) 3009–3014.
- [7] M.J. Morris, H.I. Scher, Novel strategies and therapeutics for the treatment of prostate carcinoma, Cancer 89 (2000) 1329–1348.
- [8] F. Boccardo, Hormone therapy of prostate cancer: is there a role for antiandrogen monotherapy? Crit. Rev. Oncol. Hematol. 35 (2000) 121–132.
- [9] P. Boasberg, J. Harbaugh, M. Eisenberger, J. Harris, A. Langleben, F. Ahmann, Marimastat in patients with hormone refractory prostate cancer: a dose-finding study, Proc. Am. Soc. Clin. Oncol. 16 (1997) 1126 (Abstract).
- [10] J.D. Knox, L. Bretton, T. Lynch, G.T. Bowden, R.B. Nagle, Synthetic matrix metalloproteinase inhibitor, BB-94, inhibits the invasion of neoplastic human prostate cells in a mouse model, Prostate 35 (1998) 248–254.
- [11] M. Yamaoka, T. Yamamoto, S. Ikeyama, K. Sudo, T. Fujita, Angiogenesis inhibitor TNP-470 (AGM-1470) potently inhibits the tumor growth of hormone-independent human breast and prostate carcinoma cell lines, Cancer Res. 53 (1993) 5233–5236.
- [12] J. Folkman, Angiogenesis in cancer, vascular, rheumatoid and other disease, Nat. Med. 1 (1995) 27–31.
- [13] J. Folkman, Tumor angiogenesis: therapeutic implications, N. Engl. J. Med. 285 (1971) 1182–1186.
- [14] T. Dorai, C.A. Olsson, A.E. Katz, R. Buttyan, Development of a hammerhead ribozyme against bcl-2. I. Preliminary evaluation of a potential gene therapeutic agent for hormone-refractory human prostate cancer, Prostate 32 (1997) 246–258.
- [15] K.J. Pienta, N.M. Nguyen, J.E. Lehr, Treatment of prostate cancer in the rat with the synthetic retinoid fenretinide, Cancer Res. 53 (1993) 224–226.
- [16] M. Pollard, P.H. Luckert, M.B. Sporn, Prevention of primary prostate cancer in Lobund-Wistar rats by *N*-(4-hydroxyphenyl)retinamide, Cancer Res. 51 (1991) 3610–3611.
- [17] W.K. Kelly, T. Curley, C. Liebertz, B. Kim, H.I. Scher, Phase II trial of 13-*cis*-retinoic acid and interferon- α 2a in patients with adenocarcinoma of the prostate, Proc. Am. Soc. Clin. Oncol. 15 (1996) 250 (Abstract).

- [18] G.J. Miller, G.E. Stapleton, J.A. Ferrara, M.S. Lucia, Pfister, T.E. Hedlund, P. Upadhy, The human prostatic carcinoma cell line LNCaP expresses biologically active, specific receptors for 1 α ,25-dihydroxyvitamin D₃, *Cancer Res.* 52 (1992) 515–520.
- [19] G.J. Miller, G.E. Stapleton, T.E. Hedlund, K.A. Moffat, Vitamin D receptor expression, 24-hydroxylase activity, and inhibition of growth by 1 α ,25-dihydroxyvitamin D₃ in seven human prostatic carcinoma cell lines, *Clin. Cancer Res.* 1 (1995) 997–1003.
- [20] D.M. Peehl, R.J. Skowronski, G.K. Leung, S.T. Wong, T.A. Stamey, D. Feldman, Antiproliferative effects of 1,25-dihydroxyvitamin D₃ on primary cultures of human prostatic cells, *Cancer Res.* 54 (1994) 805–810.
- [21] C. Gross, T. Stamey, S. Hancock, D. Feldman, Treatment of early recurrent prostate cancer with 1,25-dihydroxyvitamin D₃ (calcitriol), *J. Urol.* 159 (1998) 2035–2039.
- [22] T. Kubota, K. Koshizuka, E.A. Williamson, H. Asou, J.W. Said, S. Holden, I. Miyoshi, H.P. Koeffler, Ligand for peroxisome proliferator-activated receptor γ (troglitazone) has potent antitumor effect against human prostate cancer both in vitro and in vivo, *Cancer Res.* 58 (1998) 3344–3352.
- [23] P. Tontonoz, S. Singer, B.M. Forman, P. Sarraf, J.A. Fletcher, C.D. Fletcher, R.P. Brun, E. Mueller, S. Altio, H. Oppenheim, R.M. Evans, B.M. Spiegelman, Terminal differentiation of human liposarcoma cells induced by ligands for peroxisome proliferator-activated receptor γ and the retinoid X receptor, *Proc. Natl. Acad. Sci. U.S.A.* 94 (1997) 237–241.
- [24] P. Tontonoz, E. Hu, B.M. Spiegelman, Stimulation of adipogenesis in fibroblasts by PPAR γ 2, a lipid-activated transcription factor, *Cell* 79 (1994) 1147–1156.
- [25] E. Mueller, P. Sarraf, P. Tontonoz, R.M. Evans, K.J. Martin, M. Zhang, C. Fletcher, S. Singer, B.M. Spiegelman, Terminal differentiation of human breast cancer through PPAR γ , *Mol. Cell* 1 (1998) 465–470.
- [26] M. Smith, E. Mueller, G. Demetri, D. Kaufman, W. Oh, J. Jacobsen, Preliminary results: phase II trial of troglitazone for androgen-dependent (AD) and androgen-independent (AI) prostate cancer, *Proc. Am. Soc. Clin. Oncol.* 18 (1999) 328 (abstract).
- [27] N.R. Jana, S. Sarkar, M. Ishizuka, J. Yonemoto, C. Tohyama, H. Sone, Cross-talk between 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and testosterone signal transduction pathways in LNCaP prostate cancer cells, *Biochem. Biophys. Res. Commun.* 256 (1999) 462–468.
- [28] S. Safe, Modulation of gene expression and endocrine response pathways by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and related compounds, *Pharmacol. Therap.* 67 (1995) 247–281.
- [29] A. Poland, J.C. Knutson, 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin and related halogenated aromatic hydrocarbons. Examinations of the mechanism of toxicity, *Annu. Rev. Pharmacol. Toxicol.* 22 (1982) 517–554.
- [30] M.S. Denison, S.D. Seidel, W.J. Rogers, M. Ziccardi, G.M. Winter, S. Heath-Pagliuso, Natural and synthetic ligands for the Ah receptor, in: A. Puga, R.J. Kendall, (Eds.), *Molecular Biology Approaches to Toxicology*, Taylor and Francis, London, 1998, pp. 3–33.
- [31] R.J. Kociba, D.G. Keyes, J.E. Beger, R.M. Carreon, C.E. Wade, D.A. Dittenber, R.P. Kalnins, L.E. Frauson, C.L. Park, S.D. Barnard, R.A. Hummel, C.G. Humiston, Results of a 2-year chronic toxicity and oncogenicity study of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in rats, *Toxicol. Appl. Pharmacol.* 46 (1978) 279–303.
- [32] T. Zacharewski, S. Safe, Antiestrogenic activity of TCDD and related compounds, in: K.S. Korach (Ed.), *Reproductive and Developmental Toxicology*, Marcel Dekker, Inc., New York, 1998, pp. 431–448.
- [33] W. Porter, S. Safe, Estrogenic and antiestrogenic compounds, in: A. Puga, K.B. Wallace (Eds.), *Molecular Biology Approaches to Toxicology*, Techbooks, Fairfax, VA, 1998, pp. 267–283.
- [34] S. Safe, 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and related environmental antiestrogens: characterization and mechanism of action, in: R.K. Naz (Ed.), *Endocrine Disruptors*, CRC Press, Boca Raton, FL, 1999, pp. 187–221.
- [35] A. Koliopanos, J. Kleeff, Y. Xiao, S. Safe, A. Zimmerman, M.W. Buchler, H. Friess, Increased aryl hydrocarbon receptor expression offers a potential therapeutic target in pancreatic cancer, *Oncogene* 21 (2002) 6059–6070.
- [36] S. Safe, C. Qin, A. McDougal, Development of selective aryl hydrocarbon receptor modulators (SARMs) for treatment of breast cancer, *Expert Opin. Investigat. Drugs* 8 (1999) 1385–1396.
- [37] S. Safe, A. McDougal, M.S. Gupta, K. Ramamoorthy, Selective Ah receptor modulators (SAhRMs): progress towards development of a new class of inhibitors of breast cancer growth, *J. Women's Cancer* 3 (2001) 37–45.
- [38] R. Bannister, L. Biegel, D. Davis, B. Astroff, S. Safe, 6-Methyl-1,3,8-trichlorodibenzofuran (MCDF) as a 2,3,7,8-tetrachlorodibenzo-*p*-dioxin antagonist in C57BL/6 mice, *Toxicology* 54 (1989) 139–150.
- [39] C. Yao, S. Safe, 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin-induced porphyria in genetically inbred mice: partial antagonism and mechanistic studies, *Toxicol. Appl. Pharmacol.* 100 (1989) 208–216.
- [40] B. Astroff, T. Zacharewski, S. Safe, M.P. Arlotto, A. Parkinson, P. Thomas, W. Levin, 6-Methyl-1,3,8-trichlorodibenzofuran as a 2,3,7,8-tetrachlorodibenzo-*p*-dioxin antagonist: inhibition of the induction of rat cytochrome P-450 isozymes and related monooxygenase activities, *Mol. Pharmacol.* 33 (1988) 231–236.
- [41] M. Harris, T. Zacharewski, B. Astroff, S. Safe, Partial antagonism of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-mediated induction of aryl hydrocarbon hydroxylase by 6-methyl-1,3,8-trichlorodibenzofuran: mechanistic studies, *Mol. Pharmacol.* 35 (1989) 729–735.
- [42] T. Zacharewski, M. Harris, L. Biegel, V. Morrison, M. Merchant, S. Safe, 6-Methyl-1,3,8-trichlorodibenzofuran (MCDF) as an antiestrogen in human and rodent cancer cell lines: evidence for the role of the Ah receptor, *Toxicol. Appl. Pharmacol.* 13 (1992) 311–318.
- [43] G. Sun, S. Safe, Antiestrogenic activities of alternate substituted polychlorinated dibenzofurans in MCF-7 human breast cancer cells, *Cancer Chemother. Pharmacol.* 40 (1997) 239–244.
- [44] R. Dickerson, L. Howie-Keller, S. Safe, Alkyl polychlorinated dibenzofurans and related compounds as antiestrogens in the female rat uterus: structure-activity studies, *Toxicol. Appl. Pharmacol.* 135 (1995) 287–298.
- [45] A. McDougal, C. Wilson, S. Safe, Inhibition of 7,12-dimethylbenz[*a*]anthracene-induced rat mammary tumor growth by aryl hydrocarbon receptor agonists, *Cancer Lett.* 120 (1997) 53–63.
- [46] A. McDougal, M. Wormke, J. Calvin, S. Safe, Tamoxifen-induced antitumorigenic/antiestrogenic action synergized by a selective Ah receptor modulator, *Cancer Res.* 61 (2001) 3901–3907.
- [47] W. Wang, W. Porter, R. Burghardt, S. Safe, Mechanism of inhibition of MDA-MB-468 breast cancer cell growth by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, *Carcinogenesis* 18 (1997) 925–933.
- [48] R. Snoek, N. Bruchofsky, S. Kasper, R.J. Matusik, M. Gleave, N. Sato, N.R. Mawji, P.S. Rennie, Differential transactivation by the androgen receptor in prostate cancer cells, *Prostate* 36 (1998) 256–263.
- [49] J.L. Dai, C.A. Maiorino, P.J. Gkonos, K.L. Burnstein, Androgenic up-regulation of androgen receptor cDNA expression in androgen-independent prostate cancer cells, *Steroids* 61 (1996) 531–539.
- [50] K.W. Gaido, S.C. Maness, D.P. McDonnell, S.S. Dehal, D. Kupfer, S. Safe, Interaction of methoxychlor and related compounds with estrogen receptor alpha and beta and androgen receptor: structure-activity studies, *Mol. Pharmacol.* 58 (2000) 852–858.
- [51] B. Saville, M. Wormke, F. Wang, T. Nguyen, E. Enmark, G. Kuiper, J.-A. Gustafsson, S. Safe, Ligand-, cell- and estrogen receptor subtype (α/β)-dependent activation at GC-rich (Sp1) promoter elements, *J. Biol. Chem.* 275 (2000) 5379–5387.
- [52] B. Saville, Mechanisms of Estrogen Receptor (ER) Action in Breast Cancer Cells: Role of ER AF-1 Domain in ER/Sp-1 Transactivation and Modulation of ER by the Ring Finger Coactivator SNURF, Ph.D. Dissertation, Texas A&M University, 2002.
- [53] J. LaPoint, C. Labrie, Identification and cloning of a novel androgen-responsive gene, uridine diphosphoglucose, dehydrogenase in human breast cancer cells, *Endocrinology* 140 (1999) 4486–4493.

- [54] A. Poland, E. Glover, A.S. Kende, Stereospecific, high affinity binding of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin by hepatic cytosol: evidence that the binding species is receptor for induction of aryl hydrocarbon hydroxylase, *J. Biol. Chem.* 251 (1976) 4936–4946.
- [55] S. Gradelet, P. Astorg, T. Pineau, M.C. Canivenc, M.H. Siess, J. Leclerc, P. Lesca, 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 (1997) 307–315.
- [56] P.H. Jellinck, P.G. Forkert, D.S. Riddick, A.B. Okey, J.J. Michnovicz, H.L. Bradlow, Ah receptor binding properties of indole carbinols and induction of hepatic estradiol hydroxylation, *Biochem. Pharmacol.* 43 (1993) 1129–1136.
- [57] L.F. Bjeldanes, J.Y. Kim, K.R. Grose, J.C. Bartholomew, C.A. Bradfield, 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. U.S.A.* 88 (1991) 9543–9547.
- [58] I. Chen, S. Safe, L. Bjeldanes, Indole-3-carbinol and diindolylmethane as aryl hydrocarbon (Ah) receptor agonists and antagonists in T47D human breast cancer cells, *Biochem. Pharmacol.* 51 (1996) 1069–1076.
- [59] R.F. Casper, M. Quesne, I.M. Rogers, T. Shirota, A. Jolivet, E. Milgrom, J.F. Savouret, Resveratrol has antagonist activity on the aryl hydrocarbon receptor: implications for prevention of dioxin toxicity, *Mol. Pharmacol.* 56 (1999) 784–790.
- [60] S. Safe, I. Chen, W. Porter, Dietary indoles with antiestrogenic activity in common vegetables and their implications, in: E. Pavlik (Ed.), *Estrogens, Progestins and Their Antagonists*, Birkhauser, Boston, 1996, pp. 73–97.
- [61] M.A. Morse, S.D. LaGreca, S.G. Amin, F.L. Chung, Effects of indole-3-carbinol on lung tumorigenesis and DNA methylation induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and on the metabolism and disposition of NNK in A/J mice, *Cancer Res.* 50 (1990) 1613–1617.
- [62] C.J. Grubbs, V.E. Steele, T. Casebolt, M.M. Juliana, I. Eto, L.M. Whitaker, K.H. Dragnev, G.J. Kelloff, R.L. Lubet, Chemoprevention of chemically-induced mammary carcinogenesis by indole-3-carbinol, *Anticancer Res.* 15 (1995) 709–716.
- [63] I. Chen, A. McDougal, F. Wang, S. Safe, Aryl hydrocarbon receptor-mediated antiestrogenic and antitumorigenic activity of diindolylmethane, *Carcinogenesis* 19 (1998) 1631–1639.
- [64] N.R. Jana, S. Sarkar, M. Ishizuka, J. Yonemoto, C. Tohyama, H. Sone, Comparative effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on MCF-7, RL95-2, and LNCaP cells: role of target steroid hormones in cellular responsiveness to CYP1A1 induction, *Mol. Cell. Biol. Res. Commun.* 4 (2000) 174–180.
- [65] B.J. Roberts, M.L. Whitelaw, Degradation of the basic helix-loop-helix/Per-ARNT-Sim homology domain dioxin receptor via the ubiquitin/proteasome pathway, *J. Biol. Chem.* 274 (1999) 36351–36356.
- [66] Q. Ma, K.T. Baldwin, 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin-induced degradation of aryl hydrocarbon receptor (AhR) by the ubiquitin-proteasome pathway. Role of the transcription activator and DNA binding of AhR, *J. Biol. Chem.* 275 (2000) 8432–8438.
- [67] R.S. Pollenz, E.R. Barbour, Analysis of the complex relationship between nuclear export and aryl hydrocarbon receptor-mediated gene regulation, *Mol. Cell. Biol.* 20 (2000) 6095–6104.
- [68] M. Santostefano, S. Safe, Characterization of the molecular and structural properties of the transformed and nuclear aryl hydrocarbon (Ah) receptor complexes by proteolytic digestion, *Chem. Biol. Interact.* 100 (1996) 221–240.
- [69] P.S. Rennie, N. Bruchovsky, K.J. Leco, P.C. Sheppard, S.A. McQueen, H. Cheng, R. Snoek, A. Hamel, M.E. Bock, B.S. MacDonald, B.E. Nickel, C. Chang, S. Liao, P.A. Cattini, R.J. Matusi, Characterization of two *cis*-acting DNA elements involved in the androgen regulation of the probasin gene, *Mol. Endocrinol.* 7 (1993) 23–36.
- [70] M. Wormke, M. Stoner, B. Saville, K. Walker, M. Abdelrahim, R. Burghardt, S. Safe, The aryl hydrocarbon receptor mediates degradation of the estrogen receptor α through activation of proteasomes, *Mol. Cell. Biol.* 23 (2003) 1843–1855.
- [71] J.N. Davis, O. Kucuk, F.H. Sarkar, Expression of prostate-specific antigen is transcriptionally regulated by genistein in prostate cancer cells, *Mol. Carcinogen.* 34 (2002) 91–101.
- [72] B.T. Montgomery, C.Y. Young, D.L. Bilhartz, P.E. Andrews, J.L. Prescott, N.F. Thompson, D.J. Tindall, Hormonal regulation of prostate-specific antigen (PSA) glycoprotein in the human prostatic adenocarcinoma cell, LNCaP, *Prostate* 21 (1992) 63–73.
- [73] T. Furutani, T. Watanabe, K. Tanimoto, T. Hashimoto, H. Koutoku, M. Kudoh, Y. Shimizu, S. Kato, H. Shikama, Stabilization of androgen receptor protein is induced by agonist, not by antagonists, *Biochem. Biophys. Res. Commun.* 294 (2002) 779–784.
- [74] L. Sheflin, B. Keegan, W. Zhang, S.W. Spaulding, Inhibiting proteasomes in human HepG2 and LNCaP cells increases endogenous androgen receptor levels, *Biochem. Biophys. Res. Commun.* 275 (2000) 144–150.

ARYL HYDROCARBON RECEPTOR AGONISTS INHIBIT HORMONE-INDUCED TRANSACTIVATION IN PROSTATE CANCER CELLS.

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2, 3, 7, 8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and 6-methyl-1, 3, 8-trichlorodibenzofuran (6-MCDF) are ligands for the aryl hydrocarbon receptor (AhR), where 6-MCDF is a weak AhR agonist for several TCDD-like toxic responses. This study investigated the interactive effects of AhR ligands and steroid hormones on receptor proteins, hormone-induced transcriptional activation, and proliferative responses in human prostate (LNCaP and 22RV1) cancer cells. Both LNCaP and 22RV1 cells express the androgen receptor (AR), and after treatment with 10 nM dihydrotestosterone (DHT), significant 2- to 4-fold upregulation of immunoreactive AR protein is observed within 3 h, and remains elevated for up to 24 h. DHT-induced upregulation of AR is inhibited in cells treated with TCDD or 6-MCDF. In prostate cancer cells transfected with a construct (PB-luc) containing an androgen-responsive probasin gene promoter insert, both TCDD and 6-MCDF significantly inhibited DHT-induced reporter activity, whereas this activity was not inhibited in cells transfected with a construct containing only the strongly androgen responsive region (ARR3TK-luc). Ten nM 17 β -estradiol (E2) also upregulated AR levels in LNCaP and 22RV1 prostate cancer cells and E2 significantly induced reporter gene (luciferase) activity in cells transfected with PB-luc or ARR3TK-luc. TCDD and 6-MCDF inhibited E2-induced activity only in cells transfected with PB-luc. E2-induced activity in cells transfected with PB-luc was inhibited by the estrogen receptor β (ER β) antagonist 2, 2-bis[*p*-hydroxyphenyl]-1, 1, 1-trichloroethane (HPTE) and the pure antiestrogen ICI 182, 780, whereas the latter compound did not inhibit DHT-mediated transactivation. These studies indicate that AhR-AR crosstalk is dependent on promoter context (i.e. PB vs. ARR3TK). Moreover, our results also suggest that E2-responsiveness of probasin may be ER β -dependent and the inhibitory effects of TCDD and 6-MCDF indicate that the AhR may inhibit ER β -induced genes in prostate cancer cells. (Supported by DAMD17-02-1-0147 and NIH ES09106)

CROSS TALK BETWEEN DIOXIN AND HYPOXIA SENSING PATHWAYS: A GENE ARRAY STUDY.

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Although the cellular responses to 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD) and the environmental stress of hypoxia are well studied investigations of potential cross talk are limited. The endothelial response to these combined stresses is unknown. Aryl hydrocarbon receptor nuclear translocator is a common dimerization partner for both aryl hydrocarbon receptor and hypoxia inducible factor. Possible cross talk between these two pathways was studied using gene array technology. Human lung microvascular endothelial cells were exposed to 10nM TCDD, hypoxia (0%O₂) or both TCDD and hypoxia for 24 hrs and resulting RNA was analyzed using Affymetrix gene arrays. Statistical analysis of triplicate studies was performed using Affymetrix microarray suite and Affymetrix data mining tool. Ten genes were upregulated during exposure to TCDD alone and 194 genes were upregulated during exposure to hypoxia alone. A total of 147 genes were upregulated during exposure to both TCDD and hypoxia combined. The 6 genes that were upregulated only during TCDD exposure (i.e. hypoxia prevented their upregulation) encoded proteins involved in oxidant stress response, certain growth factors and cellular receptors. The 76 genes that were upregulated only during hypoxia (i.e. TCDD prevented their upregulation) encoded proteins involved in cell adhesion, extracellular matrix formation, histone binding and certain cellular receptors. The common genes upregulated under TCDD alone and in combination with hypoxia represented members of the cytochrome p450 family. The common genes upregulated under hypoxia alone and in combination with TCDD represented a wide variety of genes involved in the endothelial hypoxic response. These results demonstrate that there is cross talk between the TCDD and hypoxia sensing pathways.

ARYL HYDROCARBON RECEPTOR-MEDIATED INHIBITION OF ESTROGEN RECEPTOR-NEGATIVE BREAST CANCER CELL GROWTH.

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The mechanisms of inhibitory aryl hydrocarbon receptor (AhR)-estrogen receptor α (ER α) crosstalk in breast cancer cells have been extensively investigated, and selective AhR modulators (SAHRMs) have been developed for inhibition of ER-positive breast cancer cell/tumor growth. MDA-MB-453 and BT-474 human breast cancer cells are ER-negative and their growth is due, in part, to overexpression of

the oncogene, epidermal growth factor receptor 2 (EGFR2/ErB2/neu). In these cell lines, there is constitutive activation of the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3-K) signaling pathways. Treatment of these cells with 1 or 10 nM 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD) induces CYP1A1- or dioxin response element (DRE)-dependent activities confirming that both cell lines are Ah-responsive. Treatment of these cells with 25 μ M LY294002 or 10 μ M U1026 which inhibit PI3-K or MAPK signaling also inhibits growth of both cell lines. Growth inhibition of these cells is also observed after treatment with 10 nM TCDD, 1 - 5 μ M 6-methyl-1, 3, 8-trichlorodibenzofuran (6-MCDF) and other SAHRMs. These AhR-mediated growth inhibitory responses are also accompanied by alterations of cell cycle progression. The effects of TCDD and SAHRMs on MAPK and PI3-K signaling pathways were investigated by determining phosphorylation of MAPK and Akt. After short term (3 hr) treatment with LY294002 or U1026, there was significant inhibition of both MAPK- and PI3-K-dependent phosphorylation, whereas inhibitory effects were not observed for SAHRMs. In contrast, after treatment for up to 72 hr, SAHRMs significantly decreased activity of both kinases pathways, and the mechanisms of this response are currently being investigated. (Supported by NIH ES09106 and ES04176)

TCDD INDUCES A SUPPRESSION OF PPAR γ EXPRESSION THAT INHIBITS ADIPOCYTE DIFFERENTIATION.

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C3H10T1/2 cells (10T1/2) are a multipotential mouse embryo fibroblast cell line that can differentiate into adipocytes when administered insulin, dexamethasone, and isobutylmethylxanthine at 100% confluency; the PPAR γ ligand BRL46593 increases this differentiation. We have previously shown that PPAR γ expression is inhibited and subsequent lipid droplet accumulation is greatly diminished if TCDD is administered 48 hours prior to hormonal stimulation. Here we demonstrate that changes in TCDD-induced AhR activation positively correlate with TCDD-mediated inhibition of PPAR γ . The down regulation of AhR induction of Cyp1B1, suppression of PPAR γ expression, and suppression of the transformation into adipocytes each show a similar dependence on TCDD concentration (EC₅₀ $\sim$ 2 - 5 μ M). TCDD suppressed PPAR γ in an equally effective manner whether it was added 48 hours prior to or up to 12 hours after hormonal stimulation; however, if TCDD is administered 24 hours after the hormonal stimulation, suppression of PPAR γ does not occur. We have also shown that the PPAR γ suppression, whether TCDD treatment was initiated 48 hours prior to or 12 hours after hormonal stimulation, depended on MEK/ERK activation within the same time period of 6 - 16 hours post hormonal administration. The AhR antagonist 3'-methoxy-4'-nitroflavone (3-MNF) similarly antagonizes TCDD activation of AhR, as measured by inhibition of Cyp1B1 protein expression and suppression of PPAR γ expression. In order for 3-MNF to antagonize the TCDD effect on PPAR γ , 3-MNF must be present during the period of TCDD/MEK activity prior to the onset of PPAR γ stimulation. We conclude that TCDD induces a protein that, in conjunction with MEK activity, suppresses PPAR γ expression.

TCDD INDUCES INCREASED EXPRESSION OF RETINOIC ACID METABOLIZING GENES: POSSIBLE ROLE IN ALTERING PROLIFERATION AND DIFFERENTIATION IN HUMAN KERATINOCYTES.

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The aryl hydrocarbon receptor (AhR), is thought to mediate most of the carcinogenic and toxic effects of TCDD and related chemicals through the formation of a heterodimer with its DNA binding partner, ARNT and transcriptional regulation of AHR/ARNT target genes. We have previously shown that administration of TCDD to primary, human keratinocytes results in an immediate increase in proliferation, and a decrease in replicative senescence and a subsequent increase in late differentiation. In an effort to identify the gene pathways that regulate proliferation and differentiation and may be modified by TCDD and the AHR/ARNT heterodimer, we have performed similar experiments and analyzed the TCDD-induced changes in gene expression using microarray and western blot analysis. We have found that TCDD induces the mRNA expression levels of a number of genes involved in retinoic acid metabolism (i.e., retinol and retinoic acid dehydrogenases) in a time dependent manner. In addition, significant changes in retinoic acid receptor (RAR) isoforms were observed in the TCDD-treated cells. Given that retinoic acid is a powerful modulator of proliferation and differentiation, we suggest that the ability of TCDD to alter proliferation and differentiation in these cells may involve the alteration of retinoic acid homeostasis.

**Structure-Dependent Androgen Receptor
Agonist/Antagonist Activities and AR Downregulation by
Dichloro- and Dibromo-Substituted 1,1-Bis(3'-indolyl)methanes**

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Running Title:

Structure-dependent AR agonist/antagonist activities of substituted DIMs

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SUMMARY

1,1-Bis(3'-indolyl)methane (DIM) exhibits antiandrogenic activity in LNCaP prostate cancer cells, and this study reports the structure-dependent androgenic/antiandrogenic activity of several symmetrical dichloro- and dibromoDIM isomers. Initial transactivation studies in LNCaP and 22Rv1 cells transfected with an androgen responsive construct (pPB) containing a probasin promoter insert showed that both 7,7'-dichloro- and 7,7'-dibromoDIMs exhibited partial androgenic activity. Most of the other isomeric substituted DIMs, including 4,4'-dichloroDIM and 4,4'-dibromoDIM, exhibited antiandrogenic activity in the transactivation assay. Structure-dependent differences were also observed for the effects of 4,4'- and 7,7'-dihaloDIMs on AR expression in LNCaP cells. Like DIM, 4,4'-dichloroDIM and 4,4'-dibromoDIM did not affect AR protein levels for up to 48 hr and inhibited dihydrotestosterone (DHT)-induced responses without affecting cytosolic or nuclear AR distribution. In contrast, the AR agonist activity of 7,7'-dihaloDIMs was significantly decreased after 48 hr, and this was due to decreased AR mRNA and AR protein levels, and the latter response was proteasome-independent. Results of this study demonstrate that the antiandrogenic activity of symmetrical dihaloDIMs was structure-dependent and the 7,7'-dihaloDIMs exhibited partial AR agonist activity, whereas 4,4'-, 5,5'- and 6,6'-dihaloDIMs and DIM were antiandrogens in transactivation assays. The mechanisms of action of ring-substituted DIMs were also structure-dependent since 4,4'- and 5,5'-dihaloDIMs and DIM did not affect AR expression, and 6,6'- and 7,7'-dihaloDIMs induced degradation of AR protein and AR mRNA levels.

Key Words:

Substituted DIMs, AR agonists/antagonists, AR degradation

INTRODUCTION

Epidemiological studies have demonstrated that high consumption of cruciferous vegetables such as cauliflower, broccoli and Brussels sprouts are associated with decreased risks for several cancers [1-6]. Indole-3-carbinol (I3C) glucosinolates are expressed in high levels in *Brassica* vegetables, and the anticarcinogenic activity of these vegetables or their extracts in laboratory animal studies is associated with I3C and related chemoprotective phytochemicals [7-14]. I3C conjugates are rapidly hydrolyzed in the acidic environment of the gut and converted into structurally diverse condensation products including 1,1-bis(3'-indolyl)methane (DIM) [15, 16]. At low pH, the percentage conversion of I3C into DIM is minimal; however in cell culture studies at pH 6.6 - 7.5, I3C is primarily converted into DIM [17] and both compounds induce many of the same responses *in vitro*.

I3C and DIM exhibit anticarcinogenic activity in several animal models [7-14, 18, 19]. For example, in mice bearing mouse TRAMP-C2 prostate cancer cells as xenografts, 2.5, 5.0, and 10.0 mg/kg (3X weekly) DIM significantly decreased tumor weight/volume and induced apoptosis in the prostate tumors [19], and *in vitro* studies also show that DIM induced apoptosis in prostate cancer cells [20]. Results from several laboratories show that decreased cancer cell survival after treatment with DIM or I3C may be related to activation of multiple and possibly overlapping pathways including modulation of cell cycle regulatory proteins, induction of apoptosis and ER stress, and decreased mitochondrial membrane potential [20-29].

Le and coworkers [30] reported that DIM is a potent androgen receptor (AR) antagonist in LNCaP prostate cancer cells and inhibits dihydrotestosterone (DHT)-

induced cell proliferation and gene/reporter gene expression. Their results showed that the antiandrogenic activity of DIM was associated with the inhibition of DHT-induced AR nuclear translocation, whereas DIM alone did not induce accumulation of nuclear AR. These results were unique for DIM since other structural classes of antiandrogens such as casodex induce accumulation of nuclear AR, which is transcriptionally inactive.

Previous studies in this laboratory have shown that several ring- substituted DIMs were more potent than DIM as inhibitors of carcinogen-induced mammary tumor growth in Sprague-Dawley rats [31, 32]. The major objective of this study was to determine the structure-dependent antiandrogenic activities of a series of ring-substituted DIMs and identify potential new antiandrogens for treatment of prostate cancer. A series of symmetrical dichloro- and dibromoDIM isomers were investigated as antiandrogens and androgens in AR-responsive LNCaP and 22Rv1 prostate cancer cells transfected with the androgen-responsive pPB construct, which contains the -288 to +28 region of the probasin gene promoter linked to the luciferase gene [33, 34]. Most of the ring-substituted DIMs exhibited antiandrogenic activity; 7,7'-dichloro- and 7,7'-dibromoDIMs were only partial AR antagonists, and the compounds alone exhibited partial androgenic activity in the transactivation assay. The antiandrogenic/androgenic responses of 4,4'- and 7,7'-dihaloDIMs were further investigated by determining their effects on AR levels and expression of the androgen-responsive FKBP51 protein in LNCaP cells [35]. The antiandrogenic activities of 4,4'-dihaloDIMs were confirmed in these assays; however both 7,7'-dibromo- and 7,7'-dichloroDIM uniquely downregulated AR mRNA and protein levels after treatment for 48 hr.

MATERIALS AND METHODS

Cell Lines and Reagents. Human prostate carcinoma cell lines LNCaP and 22Rv1 were obtained from American Type Culture Collection (Manassas, VA). Fetal bovine serum was obtained from JRH Biosciences, Lenexa, KS. Cells were maintained in RPMI 1640 (Sigma Chemical St. Louis, MO) supplemented with 0.22% sodium bicarbonate, 0.011% sodium pyruvate, 0.45% glucose, 0.24% HEPES, 10% FBS, and 10 mL/L of 100x antibiotic antimycotic solution (Sigma). Cells were maintained at 37°C in the presence of 5% CO₂. Antibodies for AR (sc- 816), Sp1 (sc-59), and FKBP 51(sc-11514) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Reporter lysis buffer and luciferase reagent for luciferase studies were purchased from Promega (Madison, WI). β -Galactosidase (β -Gal) reagent was obtained from Tropix (Bedford, MA). Lipofectamine reagents were supplied by Invitrogen (Carlsbad, CA). Western Lightning chemiluminescence reagents were from Perkin-Elmer Life Sciences (Boston, MA). Dihydrotestosterone was purchased from Sigma. MG132 was obtained from Calbiochem (San Diego, CA) and gliotoxin was kindly provided by Dr. Alan Taylor, Atlantic Regional Laboratory, National Research Council (Halifax, Canada). The ring-substituted DIMs were prepared in this laboratory by condensation of ring-substituted indoles and formaldehyde or by self-condensation of ring-substituted indole-3-carbinols; compounds were >95% pure by gas chromatography-mass spectrometry as previously described [31, 32]. All substitute indoles were purchased from the Aldrich Chemical Co. (Milwaukee, WI).

Plasmids. The pPB reporter containing -288 to +28 region of the probasin gene promoter was kindly provided by Dr. Robert Matusik (Vanderbilt University Medical Centre).

Transfection and Luciferase Assay. Prostate cancer cells were plated in 12-well plates at 2.5×10^5 cells/well in DMEM:Ham's F-12 media supplemented with 2.5% charcoal-stripped FBS. After overnight attachment, cells were transfected with 400 ng of pPB and 50 ng of β -galactosidase reporter plasmid, using Lipofectamine reagent (Invitrogen) according to the manufacturer's recommended protocol. Cells were transfected and, after 8 - 9 hr, the transfection mix was replaced with 5% charcoal-stripped FBS media containing either vehicle (DMSO) or the indicated DIM-isomer and incubated for 36 - 38 hr. Based on results of preliminary studies showing that responses were not observed at concentrations $\leq 1.0 \mu\text{M}$, concentrations ranging from 5 - 15 (for 6,6'-dichloroDIM) or 5 - 20 μM were used. Cytotoxicity was observed at higher doses. Cells were then lysed with 100 μL of 1x reporter lysis buffer, and 30 μL of cell extract were used for luciferase and β -galactosidase assays. Lumicount (Packard, Meriden, CT) was used to quantitate luciferase and β -galactosidase activities, and the luciferase activities were normalized to β -gal activity.

Western Blot Analysis. LNCaP, and 22Rv1 prostate cells were seeded in 100 mm plates in DMEM:Ham's F-12 media containing 2.5% charcoal-stripped FBS and allowed to attach overnight, followed by treatment with either the vehicle (DMSO) or the indicated compounds for the desired time points. Nuclear and cytoplasmic fractionating kit (Pierce Biotechnology, Rockford, IL) was used to obtain the nuclear and cytoplasmic lysates. For whole cell lysates cells were scraped in 500 μL of lysis buffer [50 mM

HEPES, 0.5 M sodium chloride, 1.5 mM magnesium chloride, 1 mM EGTA, 10% v/v glycerol, 1% Triton X, and 5 μ L/ml of Protease Inhibitor Cocktail (Sigma)]. The lysates were incubated on ice for 1 - 1.5 hr with intermittent vortexing followed by centrifugation at 40,000 *g* for 10 min at 4°C. Protein levels were estimated using Bradford reagent; equal amounts of protein were diluted with loading buffer and boiled for 4 min, and loaded onto 10% SDS-polyacrylamide gel. After electrophoresis, gels were transferred to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA) using an electroblotting apparatus overnight at 4°C in transfer buffer containing 48 mM Tris-Cl, 29 mM glycine, and 0.025% SDS. The membranes were blocked with TBS [10 mM Tris-HCl (pH 8) and 150 mM sodium chloride] plus 5% milk (blotto-buffer) for 1 hr, and then incubated in primary antibody at 1:1000 dilution in blotto buffer at 4°C overnight, followed by one min washes (2X) and incubation with secondary antibody for 3 - 5 hr at 4°C. Membranes were then rinsed with water and incubated in enhanced chemiluminescence (ECL) reagents (Perkin Elmer, Boston, MA) for 1 min, removing excess ECL with paper towelette. The membrane was sealed in plastic wrap and photographed for immunoreactive bands using ECL hyperfilm.

RNA Extraction and cDNA Synthesis. Total RNA was extracted using Quiagen RNeasy Protect (Quiagen, Valencia, CA) according to the manufacturer's instruction. RNA was quantitated and 5 μ g was used for reverse transcription using the SuperScript II reverse transcriptase (Invitrogen).

Real-Time PCR. The cDNA was amplified in a real-time PCR using SYBR Green PCR master mix (Applied Biosystems, Foster City, CA) and 10 pM primers for AR. The reactions were performed in an ABI PRISM model 7700 sequence detector

(Applied Biosciences, Foster City, CA). The PCR conditions were as follows: 50°C for 2 min, 95°C for 10 min, 40 cycles at 95°C for 15 min, and 60°C for 1 min. The sequences for the AR primers were as follows: forward primer, 5' gta ccc tgg cgg cat ggt 3'; and reverse primer, 5' ccc att tcg ctt ttg aca ca 3'. The TATA binding protein (TBP) was used as a reference standard for quantitating AR mRNA. The primers for TBP are as follows: forward primer, 5' tgc aca gga gcc aag agt gaa 3', and reverse primer, 5' cac atc aca gct ccc cac ca 3'.

Statistical Analysis. Statistical differences between different groups were determined by ANOVA and Scheffe's test for significance. The data are presented as mean $\pm$ SE for at least three separate determinations for each treatment.

RESULTS

1. DIM and ring-substituted DIMs: AR agonist and antagonist activities in transactivation assays

Previous studies show that DIM exhibits AR antagonist activity in LNCaP cells and inhibits DHT-induced PSA protein and reporter gene activity in cells transfected with androgen-responsive constructs [30]. In this study, the antiandrogenic activity of DIM has been investigated in LNCaP and 22Rv1 prostate cancer cells transfected with the androgen-responsive pPB construct (Fig. 1). Both cell lines express mutant forms of the AR; however, DHT activates the receptor in LNCaP and 22Rv1 cells [36, 37]. The results show that DHT but not DIM alone significantly induced luciferase activity in LNCaP and 22Rv1 cells transfected with pPB. In cells cotreated with DHT plus DIM and transfected with pPB, hormone-induced luciferase activity was significantly decreased at DIM concentrations of 5, 10 and 20 μ M. These results confirm that DIM inhibits activation of the androgen responsive pPB construct by DHT in both LNCaP and 22Rv1 cells and complements results of a previous report on the antiandrogenic activity of DIM [30].

Ring-substituted DIMs also exhibit potent anticancer activities [31, 32]; however, the structure-dependent effects of these compounds as antiandrogens have not been reported. This study investigates the AR antagonist and agonist activities of symmetrical dihaloDIMs containing substituents in the 4, 5, 6, and 7 positions of the benzene ring. The structure-dependent AR antagonist/agonist activities of 4,4'-, 5,5'-, 6,6'-, and 7,7'-dichloro- and -dibromoDIMs were investigated in LNCaP and 22Rv1 cells transfected with the PB construct (Figs. 2 and 3). The dichloro- and dibromoDIM

isomers induce similar structure-dependent responses in both cell lines. The results obtained for the dichloroDIM isomers show that 4,4'-, 5,5'-, and 6,6'-dichloroDIM were AR antagonists in LNCaP and 22Rv1 cells (Figs. 2A and 2B), although their AR antagonist activity was more pronounced in the latter cell line. 7,7'-DichloroDIM was a partial AR agonist/antagonist in both cell lines (Figs. 2C and 2D), whereas the other isomers did not exhibit AR agonist activities. The pattern of antiandrogenic/androgenic activities for the isomeric dichloroDIMs (Fig. 2) was similar to that observed for the brominated analogs (Fig. 3). 4,4'-, 5,5'- and 6,6'-DibromoDIM primarily exhibited antiandrogenic activities in LNCaP and 22Rv1 cells (Figs. 3A and 3B), and 7,7'-dibromoDIM was a partial AR agonist/antagonist in both cell lines (Figs. 3C and 3D).

2. Structure-dependent effects of isomeric dihaloDIMs on AR protein expression

The antiandrogenic activity of DIM was associated with inhibition of DHT-induced formation of nuclear AR [30]; however, other compounds such as tea polyphenols and emodin inhibit androgen responsiveness through downregulation of AR protein [38, 39]. We therefore investigated the effects of the dichloroDIM (Fig. 4A) and dibromoDIM (Fig. 4B) isomers on AR protein expression in LNCaP cells. Cells were treated with different concentrations of the individual compounds for 24 hr and whole cell lysates were analyzed for AR protein by Western blot analysis. 4,4'- and 5,5'-DichloroDIM (up to 20 μ M) did not affect AR protein levels; however, AR protein expression was decreased by both 6,6'- and 7,7'-dichloroDIM. Results are shown only for 15 μ M 6,6'-dichloroDIM due to the high cytotoxicity of this compound. Results in Figure 4B for the dibromoDIMs gave a similar pattern of isomer-dependent responses, namely 4,4'-dibromoDIM had minimal

effects on levels of AR protein, whereas both 6,6'- and 7,7'-dibromoDIM decreased AR levels. 5,5'-DibromoDIM also decreased expression of AR protein but only at the 20 μ M concentration. These results demonstrate remarkable differences in the effects of 4,4'-/5,5'-dihaloDIMs and 6,6'-/7,7'-dihaloDIMs on AR protein expression in LNCaP cells, and these differences were further investigated using the 4,4' and 7,7'-dihaloDIM as prototypes.

Previous studies showed that nuclear levels of AR increased after treatment of LNCaP cells with DHT for 24 hr, and DHT-induced nuclear translocation of AR was inhibited after cotreatment with DIM [30]. Figure 5A summarizes the effects of 20 μ M 4,4'- and 7,7'-dichloroDIM, 20 μ M DIM and 10 nM DHT on cytosolic and nuclear AR levels after treatment for 1 and 24 hr. Minimal changes in cytosolic (c) and nuclear (n) AR levels were observed in all treatment groups (compared to DMSO) after 1 hr, and no major changes in AR protein staining in the cytosolic or nuclear fractions were observed. In cells treated for 24 hr, DHT induced a more intense staining of AR in the nuclear fraction and enhanced overall AR staining (c+n) compared to cells treated with DMSO alone (c+n). This was observed in replicate experiments and is consistent with results of previous studies showing that DHT enhanced AR expression in LNCaP cells [33, 40, 41]. Both DIM and 4,4'-dichloroDIM alone also enhanced AR levels in both the cytosolic and nuclear fractions; however, in combination with DHT, these compounds did not block DHT-induced formation of nuclear AR, and this was in contrast to a previous report showing that DIM inhibited this response[30]. In contrast, 7,7'-dichloroDIM alone decreased nuclear and cytosolic AR levels after treatment for 24 hr and, in combination (DHT + 7,7'-dichloroDIM), the DHT-induced nuclear AR levels were only slightly

decreased. The observed downregulation of AR protein in both the cytosolic and nuclear fractions (Fig. 5A) complements the results observed for AR levels in whole cell lysates from cells treated with 7,7'-dichloroDIM (Fig. 5A). Sp1 protein served as a loading control for this study, and the identification of Sp1 only in the nuclear fraction confirms the efficiency of the separation of nuclear and cytosolic fractions.

In two separate experiments (1 and 24 hr), the effects of DMSO, DHT, 4,4'- and 7,7'-dibromoDIM on AR levels were determined in LNCaP cells (Fig. 5B). The pattern of effects for the dibromoDIMs alone, and in combination with DHT was similar to those observed for the dichloroDIM isomers. DHT and 4,4'-dibromoDIM induced a time-dependent increase in AR levels (compared to DMSO). Interaction of 4,4'-dibromoDIM with DHT decreased the ratio of nuclear/cytosolic levels of AR; however, this could be an additive effect since the former compounds alone induced higher cytosolic AR levels. In contrast, cytosolic and nuclear AR protein levels were decreased after treatment with 7,7'-dibromoDIM alone for 24 hr. In cells cotreated with 7,7'-dibromoDIM plus DHT, AR levels and their distribution were similar to those observed for DHT alone. Both 7,7'-dibromo- and 7,7'-dichloroDIM appeared to induce a time-dependent decrease in AR protein, whereas DHT, DIM and 4,4'-dichloro-, and 4,4'-dibromoDIM increased or stabilized AR protein in LNCaP cells. This was further investigated in LNCaP cells treated for 48 hr with DHT, 4,4'- and 7,7'-dihaloDIMs followed by Western blot analysis of whole cell lysates. Results in Figure 6A confirm that 7,7'-dichloro- and 7,7'-dibromoDIM decreased AR protein expression (compared to DHT), whereas AR levels after treatment with the corresponding 4,4'-dihaloDIMs (Fig. 6B) were significantly higher than observed in cells treated with the 7,7'-dihaloDIMs (Fig. 6A). We also

observed that Sp1 protein was also slightly decreased only after prolonged treatment with the 7,7'-dihaloDIMs.

3. Effects of dihaloDIM isomers on AR protein and mRNA levels and their antiandrogenic activities in LNCaP cells

The potential role of proteasome activation in mediating downregulation of AR was investigated. LNCaP cells were treated with 7,7'-dihaloDIMs for 48 hr in the presence or absence of the proteasome inhibitors gliotoxin or MG132 (Fig. 6C). Western blot analysis of whole cell lysates showed that 7,7'-dichloro- and 7,7'-dibromoDIM significantly decreased AR protein compared to levels observed in solvent-treated cells, and cotreatment with the proteasome inhibitors further increased AR degradation. The proteasome inhibitors alone also decreased AR protein, whereas 4,4'-dichloro- and 4,4'-dibromoDIM did not affect AR protein. The data indicated that decreased AR protein in LNCaP cells treated with 7,7'-dihaloDIMs is not due to activation of the proteasome pathway. The time-dependent effects of 7,7'-dihaloDIMs on AR mRNA levels was determined (Fig. 6D), and the results show that mRNA levels are significantly decreased within 24 and 48 hr (data not shown). We also investigated the effects of 7,7'-dichloroDIM on AR mRNA stability by pretreating cells with DMSO or 20 μ M 7,7'-dichloroDIM for 12 hr prior addition of actinomycin D. The results showed an initial 6 - 12 hr increase in AR mRNA levels after addition of actinomycin D; however, the subsequent rates of degradation of AR mRNA in the DMSO and 7,7'-dichloroDIM treatment groups were comparable (data not shown). These data indicate that 7,7'-dihaloDIMs decrease both transcriptional and translational regulation of the AR.

Results of transient transfection studies showed that both 7,7'-dibromo- and 7,7'-dichloroDIM were partial AR agonists and AR antagonists (Figs. 2 and 3), and the former was observed after treatment for 36 hr. This AR agonist activity of 7,7'-dihaloDIMs is inconsistent with their effects on AR and it is possible that 7,7'-dihaloDIM-induced androgenic activity after 36 hr (Figs. 2 and 3) may be due to the relatively slow rate of AR degradation. We therefore investigated the time-dependent effects of 7,7'-dihaloDIMs on androgen-responsiveness in LNCaP cells transfected with pPB (for 9 hr), and then treated with different concentrations of 7,7'-dihaloDIMs for 24, 36 or 48 hr (Fig. 7A). The results indicated that after 36 hr, 5 - 20 μ M 7,7'-dichloroDIM significantly induced luciferase activity; however, this response was significantly decreased after treatment for 24 or 48 hr and similar results were observed for 7,7'-dibromoDIM (data not shown). The decreased AR agonist activity of 7,7'-dichloroDIM after 48 hr is consistent with the effects of this compound on AR protein degradation (Fig. 6A).

A recent study identified a 51 kDa progesterone receptor-associated immunophilin, FKBP51, as an androgen-responsive gene in prostate cancer cells [35], and the effects of 4,4'-dichloro- and 4,4'-dibromoDIM alone and in combination with DHT were investigated in LNCaP cells 48 hr after treatment (Fig. 7B). DHT alone enhanced FKBP51 protein expression, whereas 4,4'-dichloro- and 4,4'-dibromoDIM did not affect levels of FKBP51. In cells cotreated with DHT plus 4,4'-dichloro- or 4,4'-dibromoDIM, the hormone-induced response was inhibited by both DIM compounds and this was consistent with their antiandrogenic activity in transactivation assays (Figs. 2 and 3). Minimal induction of FKBP51 was observed after treatment with 7,7'-dichloro- or 7,7'-dibromoDIM (data not shown) and this may be due, in part, to the low levels of

AR expression in LNCaP cells treatment with these compounds for 48 hr. In summary, these results indicate that ring-substituted DIMs and DIM differentially modulate androgenic responses in prostate cancer cells and subtle changes in the position of the ring substituents of the dihaloDIMs (i.e. 4 vs. 7) can modulate their mechanisms of antiandrogenic action and effects on AR expression.

DISCUSSION

I3C and DIM inhibit growth of several cancer cell lines through multiple mechanisms and these compounds also directly activate receptors. I3C and/or DIM interact with the AhR, the fish ER and AR [15-18], and ring-substituted DIMs also activated the AhR [31, 32]. A recent study showed that DIM competitively bound the AR and decreased DHT-induced transactivation/gene expression in LNCaP cells [30]. The mechanism of the antiandrogenic activity of DIM was novel since this compound inhibited DHT-induced nuclear translocation of the cytosolic AR [30], whereas other antiandrogens do not block nuclear translocation of the AR but form transcriptionally inactive nuclear AR complexes [42, 43]. This study reports the androgenic/antiandrogenic activity of a series of symmetrical ring-substituted DIMs to delineate possible structure-dependent effects that modulate their activities.

Results in Figures 2 and 3 demonstrated that like DIM (Fig. 1), the symmetrically substituted dichloro- and dibromoDIM isomers also inhibited DHT-induced transactivation in LNCaP and 22Rv1 prostate cancer cells. The assay system used the androgen responsive pPB construct, which contains the -288 to +28 region of the probasin reporter linked to firefly luciferase [33, 34]. The antiandrogenic activities of the compounds were similar in both cell lines; however, with few exceptions their overall potencies were higher in LNCaP than 22Rv1 cells. We also investigated the structure-dependent AR agonist activities of ring-substituted DIMs in cells transfected with pPB. 7,7'-Dichloro- and 7,7'-dibromoDIM were partial AR agonists and partial antagonists in both cell lines (Figs. 2 and 3), and their androgenic potencies were at least three orders of magnitude lower than that of DHT. In contrast, symmetrical 4,4'-, 5,5'-, 6,6'-dichloro-

and -dibromoDIMs primarily exhibited antiandrogenic activity in this transactivation assay in both cell lines. Other symmetrical ring-substituted methyl, methoxy and fluoroDIM analogs also exhibited antiandrogenic activity and a few of these isomers were also partial AR agonists; however, these activities also depended on cell context and were not further investigated (data not shown).

The structure-dependent effects of dihaloDIMs on AR expression clearly differentiated between the 4,4'-/5,5'- and 6,6'-/7,7'-dihaloDIMs since the latter compounds downregulated AR protein, whereas minimal effects were observed for 4,4'- and 5,5'-dihaloDIMs (Fig. 4). 6,6'-DichloroDIM exhibited significant cytotoxicity at concentrations > 10 μ M and, therefore, structure-dependent differences in the activities of the dihaloDIM isomers were further investigated using 4,4'- and 7,7'-dichloro- and -dibromoDIMs as models in LNCaP cells. Previous studies show that DHT stabilizes the AR (compared to DMSO) resulting in increased AR expression for at least 24 hr after treatment [33, 40, 41], and results of this study also showed that treatment with DHT increased AR protein (Figs. 5 and 6). We directly compared AR protein levels in LNCaP cells treated with DMSO (control), DHT, 4,4'-dichloro-, 7,7'-dichloro-, 4,4'-dibromo-, 7,7'-dibromoDIM, DIM alone, and DHT plus the DIMs (in combination) for 1 and 24 hr (Fig. 5). The most pronounced changes were observed in the 24 hr treatment group, where DHT, DIM, 4,4'-dichloro- and 4,4'-dibromoDIM stabilized the AR (compared to DMSO). DHT alone induced nuclear translocation of the AR; however, in contrast to a previous report with DIM [30], results of this study showed that DIM and 4,4'-dihaloDIMs did not markedly inhibit DHT-induced AR translocation and the combined treatments tended to give additive effects (Fig. 5). 7,7'-DichloroDIM also had

minimal effects on DHT-induced translocation of the AR; however, both 7,7'-dichloro- and 7,7'-dibromoDIM alone did not stabilize the AR protein after treatment for 24 hr.

Compared to treatment with DHT, DIM or the 4,4'-dihalo-DIMs, 7,7'-dichloro-, and 7,7'-dibromoDIM significantly decreased AR protein and mRNA levels in LNCaP cells (Fig. 6). Downregulation of AR by these compounds was similar to the reported time-dependent decrease of AR protein in LNCaP cells after treatment with tea polyphenol epigallocatechin (EGCG) [38]. It was hypothesized that the effects of EGCG were due to downregulation of Sp1 protein, which also plays a role in regulating AR expression. We also observed that the 7,7'-dihaloDIMs slightly decreased Sp1 protein expression after treatment for 48 hr (Figs. 6A and 6B). This may contribute, in part, to the lower expression of AR in these cells but cannot fully explain the dramatic drop in AR protein. A recent study reported that the phytochemical emodin inhibited AR-dependent transactivation in prostate cancer cells, and this was associated with inhibition of AR nuclear translocation and activation of proteasome-dependent degradation of AR protein [39]. The effects of emodin on AR protein in LNCaP cells were blocked by the proteasome inhibitor MG132, and emodin did not affect AR mRNA levels. In contrast 7,7'-dihaloDIM-induced degradation of AR protein was not blocked by the proteasome inhibitors gliotoxin or MG132 (Fig. 6C), and these compounds induced a time-dependent decrease in both AR mRNA and protein levels. We did not observe any compound-induced changes in AR mRNA stability (data not shown), suggesting a transcriptional mechanism of action which is being currently investigated. These results clearly distinguished between the effects of 7,7'-dihaloDIMs with other

compounds such as emodin and ECCG which also affect AR mRNA and protein expression in LNCaP cells.

Although the initial *in vitro* screening assays showed that 7,7'-dihaloDIMs exhibited potential AR agonist activity in transactivation assays, we further investigated the effects of duration of treatment on their androgenic activity. 7,7'-DihaloDIMs induced transactivation in LNCaP cells transfected with pPB and treated for 36 hr (Fig. 7A). However, after 48 hr, this response was significantly decreased and this was consistent with decreased AR expression at this time point, suggesting that the partial androgenic activity of these compounds is reversed after longer periods of exposure due to AR downregulation.

The AR agonist activity of the 7,7'-dihaloDIMs was also affected by lower AR expression since these compounds did not significantly induce FKBP protein in LNCaP cells (data not shown). The androgen-responsive FKBP protein is maximally induced by DHT only after treatment for 48 hr, and 4,4'-dichloro- and 4,4'-dibromoDIM inhibited this response (Fig. 7B).

In summary, results of this study confirm that DIM and several symmetrical ring-substituted DIM congeners exhibit antiandrogenic activity. In addition, some isomers, notably 7,7'-dichloro- and 7,7'-dibromoDIM also exhibit partial time-dependent androgenic activity in transfection assays, and these results illustrate that subtle changes in the phenyl ring substitution pattern have marked effects on the androgenic activity of the dihaloDIMs. At the concentrations used in this study, the antiandrogenic activity of the 4,4'-dihaloDIMs was not related to inhibition of DHT-induced nuclear translocation of AR. Our results suggest that the antiandrogenic activity of DIM and

4,4'-dihaloDIMs may be complex and involve multiple pathways including inhibition of nuclear AR-dependent transactivation. We also observed that 6,6'-dihaloDIMs and 7,7'-dihaloDIMs decreased AR expression in LNCaP cells, and current studies are investigating the potential clinical importance of these and other effects of ring-substituted DIMs on the growth of prostate cancer cells/tumors in both *in vitro* and *in vivo* models.

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REFERENCES

1. Murillo G, Mehta RG. Cruciferous vegetables and cancer prevention. *Nutr Cancer* 2001; 41:17-28.
2. Kristal AR, Lampe JW. *Brassica* vegetables and prostate cancer risk: a review of the epidemiological evidence. *Nutr Cancer* 2002; 42:1-9.
3. Cohen JH, Kristal AR, Stanford JL. Fruit and vegetable intakes and prostate cancer risk. *J Natl Cancer Inst* 2000; 92:61-81.
4. Kolonel LN, Hankin JH, Whittemore AS et al. Vegetables, fruits, legumes and prostate cancer: a multiethnic case-control study. *Cancer Epidemiol Biomarkers Prev* 2000; 9:795-804.
5. Zhang SM, Hunter DJ, Rosner BA et al. Intakes of fruits, vegetables, and related nutrients and the risk of non-Hodgkin's lymphoma among women. *Cancer Epidemiol Biomarkers Prev* 2000; 9:477-485.
6. Preobrazhenskaya MN, Bukhman VM, Korolev AM, Efimov SA. Ascorbigen and other indole-derived compounds from *Brassica* vegetables and their analogs as anticarcinogenic and immunomodulating agents. *Pharmacol Therap* 1993; 60:301-313.
7. Wattenberg LW, Loub WD. Inhibition of polycyclic hydrocarbon-induced neoplasia by naturally occurring indoles. *Cancer Res* 1978; 38:1410-1413.
8. Bradlow HL, Michnovicz JJ, Telang NT, Osborne MP. Effects of dietary indole-3-carbinol on estradiol metabolism and spontaneous mammary tumors in mice. *Carcinogenesis* 1991; 12:1571-1574.

9. Morse MA, LaGreca SD, Amin SG, Chung FL. Effects of indole-3-carbinol on lung tumorigenesis and DNA methylation induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and on the metabolism and disposition of NNK in A/J mice. *Cancer Res* 1990; 50:1613-1617.
10. Tanaka T, Kojima T, Morishita Y, Mori H. Inhibitory effects of the natural products indole-3-carbinol and sinigrin during initiation and promotion phases of 4-nitroquinoline 1-oxide-induced rat tongue carcinogenesis. *Jpn J Cancer Res* 1992; 83:835-842.
11. Tanaka T, Mori Y, Morishita Y et al. Inhibitory effect of sinigrin and indole-3-carbinol in diethylnitrosamine-induced hepatocarcinogenesis in male ACI/N rats. *Carcinogenesis* 1990; 11:1403-1406.
12. Grubbs CJ, Steele VE, Casebolt T et al. Chemoprevention of chemically-induced mammary carcinogenesis by indole-3-carbinol. *Anticancer Res* 1995; 15:709-716.
13. Kim DJ, Lee KK, Han BS et al. Biphasic modifying effect on indole-3-carbinol on diethylnitrosamine-induced preneoplastic glutathione S-transferase placental form-positive liver cell foci in Sprague-Dawley rats. *Jpn J Cancer Res* 1994; 85:578-583.
14. Kojima T, Tanaka T, Mori H. Chemoprevention of spontaneous endometrial cancer in female Donryu rats by dietary indol-3-carbinol. *Cancer Res* 1994; 54:1446-1449.
15. 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 U S A* 1991; 88:9543-9547.
16. Grose KR, Bjeldanes LF. Oligomerization of indole-3-carbinol in aqueous acid. *Chem Res Toxicol* 1992; 5:188-193.
 17. Niwa T, Swaneck G, Bradlow HL. Alterations in estradiol metabolism in MCF-7 cells induced by treatment with indole-3-carbinol and related compounds. *Steroids* 1994; 59:523-527.
 18. Chen I, McDougal A, Wang F, Safe S. Aryl hydrocarbon receptor-mediated antiestrogenic and antitumorigenic activity of diindolylmethane. *Carcinogenesis* 1998; 19:1631-1639.
 19. Nachshon-Kedmi M, Fares FA, Yannai S. Therapeutic activity of 3,3'-diindolylmethane on prostate cancer in an *in vivo* model. *Prostate* 2004; 61:153-160.
 20. Nachshon-Kedmi M, Yannai S, Fares FA. Induction of apoptosis in human prostate cancer cell line, PC3, by 3,3'-diindolylmethane through the mitochondrial pathway. *Br J Cancer* 2004; 91:1358-1363.
 21. Hong C, Kim HA, Firestone GL, Bjeldanes LF. 3,3'-Diindolylmethane (DIM) induces a G1 cell cycle arrest in human breast cancer cells that is accompanied by Sp1-mediated activation of p21(WAF1/CIP1) expression. *Carcinogenesis* 2002; 23:1297-1305.
 22. Cover CM, Hsieh SJ, Tran SH et al. Indole-3-carbinol inhibits the expression of cyclin-dependent kinase-6 and induces a G1 cell cycle arrest of human breast

- cancer cells independent of estrogen receptor signaling. *J Biol Chem* 1998; 273:3838-3847.
23. Ge X, Yannai S, Rennert G, Gruener N, Fares FA. 3,3'-Diindolylmethane induces apoptosis in human cancer cells. *Biochem Biophys Res Commun* 1996; 228:153-158.
 24. Rahman KM, Aranha O, Glazyrin A, Chinni SR, Sarkar FH. Translocation of Bax to mitochondria induces apoptotic cell death in indole-3-carbinol (I3C) treated breast cancer cells. *Oncogene* 2000; 19:5764-5771.
 25. Chinni SR, Li Y, Upadhyay S, Koppolu PK, Sarkar FH. Indole-3-carbinol (I3C) induced cell growth inhibition, G1 cell cycle arrest and apoptosis in prostate cancer cells. *Oncogene* 2001; 20:2927-2936.
 26. Firestone GL, Bjeldanes LF. Indole-3-carbinol and 3,3'-diindolylmethane antiproliferative signaling pathways control cell-cycle gene transcription in human breast cancer cells by regulating promoter-Sp1 transcription factor interactions. *J Nutr* 2003; 133:2448S-2455S.
 27. Hong C, Firestone GL, Bjeldanes LF. Bcl-2 family-mediated apoptotic effects of 3,3'-diindolylmethane (DIM) in human breast cancer cells. *Biochem Pharmacol* 2002; 63:1085-1097.
 28. Rahman KM, Aranha O, Sarkar FH. Indole-3-carbinol (I3C) induces apoptosis in tumorigenic but not in nontumorigenic breast epithelial cells. *Nutr Cancer* 2003; 45:101-112.

29. Sun S, Han J, Ralph WM, Jr. et al. Endoplasmic reticulum stress as a correlate of cytotoxicity in human tumor cells exposed to diindolylmethane in vitro. *Cell Stress Chaperones* 2004; 9:76-87.
30. Le HT, Schaldach CM, Firestone GL, Bjeldanes LF. Plant-derived 3,3'-diindolylmethane is a strong androgen antagonist in human prostate cancer cells. *J Biol Chem* 2003; 278:21136-21145.
31. 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* 2000; 151:169-179.
32. McDougal A, Gupta MS, Morrow D et al. Methyl-substituted diindolylmethanes as inhibitors of estrogen-induced growth of T47D cells and mammary tumors in rats. *Breast Cancer Res Treat* 2001; 66:147-157.
33. Morrow D, Qin C, Smith III R, Safe S. Aryl hydrocarbon receptor-mediated inhibition of LNCaP prostate cancer cell growth and hormone-induced transactivation. *J Steroid Biochem Mol Biol* 2004; 88:27-36.
34. Snoek R, Bruchovsky N, Kasper S et al. Differential transactivation by the androgen receptor in prostate cancer cells. *Prostate* 1998; 36:256-263.
35. Velasco AM, Gillis KA, Li Y et al. Identification and validation of novel androgen-regulated genes in prostate cancer. *Endocrinology* 2004; 145:3913-3924.
36. van Bokhoven A, Varella-Garcia M, Korch C et al. Molecular characterization of human prostate carcinoma cell lines. *Prostate* 2003; 57:205-225.

37. McDonald S, Brive L, Agus DB, Scher HI, Ely KR. Ligand responsiveness in human prostate cancer: structural analysis of mutant androgen receptors from LNCaP and CWR22 tumors. *Cancer Res* 2000; 60:2317-2322.
38. Ren F, Zhang S, Mitchell SH, Butler R, Young CY. Tea polyphenols down-regulate the expression of the androgen receptor in LNCaP prostate cancer cells. *Oncogene* 2000; 19:1924-1932.
39. Cha TL, Qiu L, Chen CT, Wen Y, Hung MC. Emodin down-regulates androgen receptor and inhibits prostate cancer cell growth. *Cancer Res* 2005; 65:2287-2295.
40. Blanchere M, Berthaut I, Portois MC, Mestayer C, Mowszowicz I. Hormonal regulation of the androgen receptor expression in human prostatic cells in culture. *J Steroid Biochem Mol Biol* 1998; 66:319-326.
41. Furutani T, Watanabe T, Tanimoto K et al. Stabilization of androgen receptor protein is induced by agonist, not by antagonists. *Biochem Biophys Res Commun* 2002; 294:779-784.
42. Georget V, Lobaccaro JM, Terouanne B et al. Trafficking of the androgen receptor in living cells with fused green fluorescent protein-androgen receptor. *Mol Cell Endocrinol* 1997; 129:17-26.
43. Masiello D, Cheng S, Bubley GJ, Lu ML, Balk SP. Bicalutamide functions as an androgen receptor antagonist by assembly of a transcriptionally inactive receptor. *J Biol Chem* 2002; 277:26321-26326.

FIGURE CAPTIONS

Figure 1. Antiandrogenic activity of DIM. LNCaP (A) or 22Rv1 (B) cells were transfected with pPB, treated with DHT, 5 - 20 μ M DIM alone or in combination with DHT, and luciferase activity determined as described in the Materials and Methods. Results are expressed as means $\pm$ SE for at least 3 determinations per treatment group, and significant ($p < 0.05$) induction (*) or inhibition (**) in the cotreatment groups are indicated.

Figure 2. Antiandrogenic and androgenic activity of isomeric dichloroDIMs in LNCaP (A, C) and 22Rv1 (B, D) cells. Cells were transfected with pPB, treated with DHT, 5 - 20 μ M dichloroDIMs alone or in combination with DHT, and luciferase activity determined as described in the Materials and Methods. Results are expressed as means $\pm$ SE for at least 3 determinations per treatment group, and significant ($p < 0.05$) induction (*) or inhibition (**) in the 20 μ M cotreatment groups are indicated.

Figure 3. Antiandrogenic and androgenic activity of isomeric dibromoDIMs in LNCaP (A, C) and 22Rv1 (B, D) cells. Cells were transfected with pPB, treated with DHT, 5 - 20 μ M dibromoDIMs alone or in combination with DHT, and luciferase activity determined as described in the Materials and Methods. Results are expressed as means $\pm$ SE for at least 3 determinations per treatment group, and significant ($p < 0.05$) induction (*) or inhibition (**) in the 20 μ M cotreatment groups are indicated.

Figure 4. Structure-dependent effects of isomeric dihaloDIMs on AR protein levels. LNCaP cells were treated with different concentrations of isomeric dichloroDIMs (A) and dibromoDIMs (B) for 24 hr, and whole cell lysates were analyzed for AR and β -actin (loading control) protein by Western blot analysis as described in the Materials and Methods.

Figure 5. Cytosolic (c) and nuclear (n) AR protein in LNCaP cells treated with DIM and dihaloDIMs. (A) Treatment with DIM, 4,4'- and 7,7'-dichloroDIM. Cells were treated with DMSO, DHT, 4,4'- and 7,7'-dichloroDIM or DIM alone or in combination with DHT for 1 or 24 hr, and cytosolic or nuclear fractions were obtained and analyzed by Western blot analysis as described in the Materials and Methods. The nuclear Sp1 protein was determined as a loading control and to determine the efficiency of the isolation of the cytosolic and nuclear fractions. (B) Treatment with DIM, 4,4'- and 7,7'-dibromoDIM. Cells treated with DMSO, DHT, 4,4' or 7,7'-dibromoDIM alone or in combination with DHT for 48 hr, and nuclear and cytosolic fractions were analyzed by Western blot analysis as described in the Materials and Methods. Nuclear Sp1 protein serves as a control for determining the efficiency of the isolated cytosolic and nuclear fractions.

Figure 6. Effects of 4,4'- or 7,7'-dihaloDIM on AR expression and androgen responsiveness. Effects of 7,7'-dihaloDIMs (A) and 4,4'-dihaloDIMs (B) on AR protein levels. LNCaP cells were treated with DMSO, 10 nM DHT, 20 μ M dihaloDIMs for 48 hr, and whole cell lysates were analyzed by Western blot analysis as described in Materials and Methods. AR, β -actin (loading control) and Sp1 protein were determined. (C)

Effects of proteasome inhibitors on AR protein levels. LNCaP cells were treated with 20 μ M 7,7'-dichloro- or 7,7'-dibromo DIM alone or in combination with the proteasome inhibitor gliotoxin (3 μ M) or MG132 (10 μ M) for 48 hr, and AR protein levels were determined by Western blot analysis as described in Materials and Methods. β -Actin served as a loading control. (D) Time-dependent effects of 7,7'-dihaloDIMs on AR mRNA protein levels. LNCaP cells were treated with 7,7'-dihaloDIMs for 24 hr, and AR mRNA was determined by real-time PCR as described in the Materials and Methods. The experiments were carried out in triplicate. Results are expressed as means $\pm$ SE, and significantly ($p < 0.05$) decreased AR mRNA is indicated by an asterisk. TBP mRNA was also determined and used to normalize the AF mRNA levels.

Figure 7. AR agonist/antagonist activities of dihaloDIMs. (A) Time-dependent effects of 7,7'-dichloroDIM on transactivation. LNCaP cells were transfected with pPB and, after 9 hr, were treated with 7,7'-dichloroDIM (5 - 20 μ M) for 24, 36 and 48 hr, and luciferase activity was determined as described in Materials and Methods. Results are expressed as means $\pm$ SE for at least 3 determinations for each treatment group and significant ($p < 0.05$) induction (*) is indicated. (B) Regulation of FKBP51 protein expression. LNCaP cells were treated with DMSO, 10 nM DHT, 4,4'-dichloro- or 4,4'-dibromoDIM alone or in combination with DHT for 48 hr, and whole cell lysates were analyzed by Western blot analysis for FKBP and β -actin (loading control) as described in Materials and Methods.

Figure 1

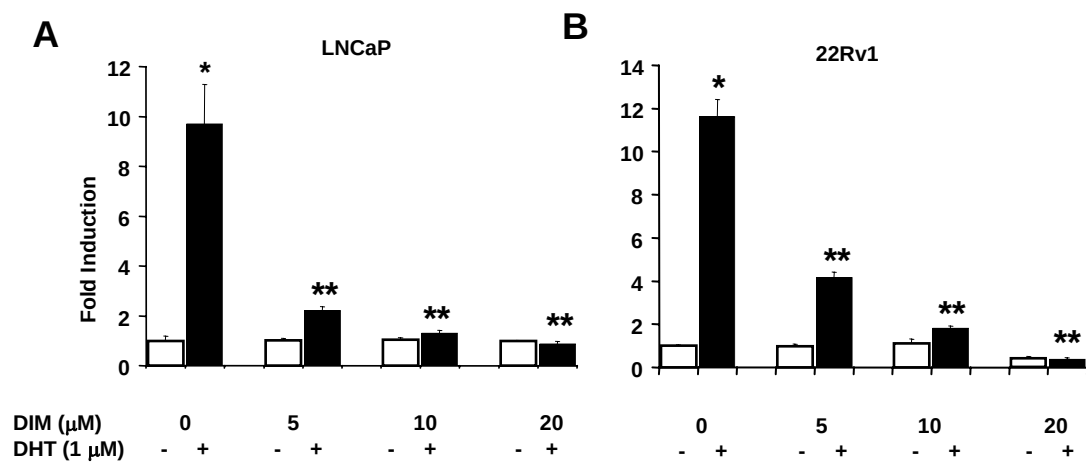


Figure 2

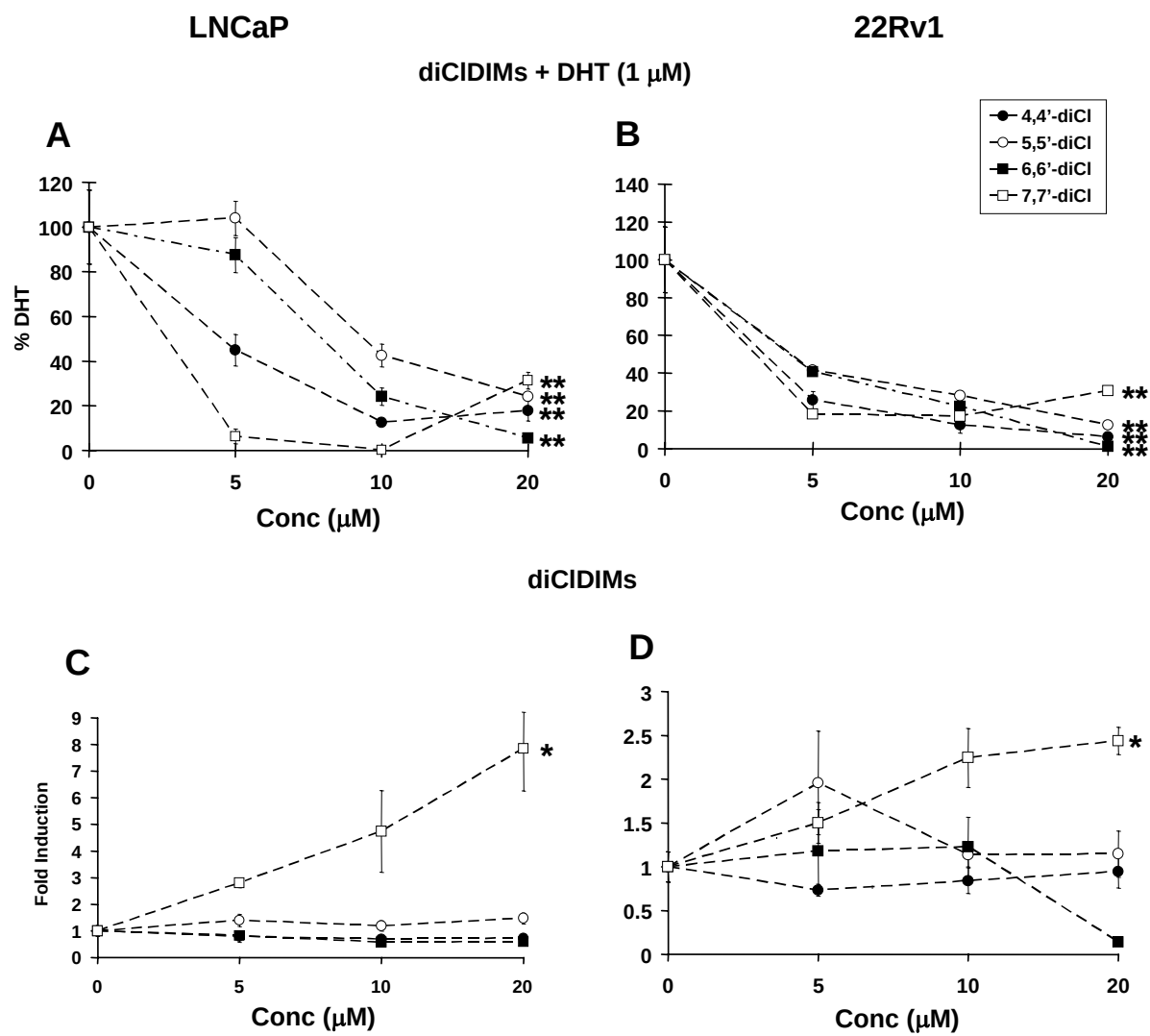


Figure 3

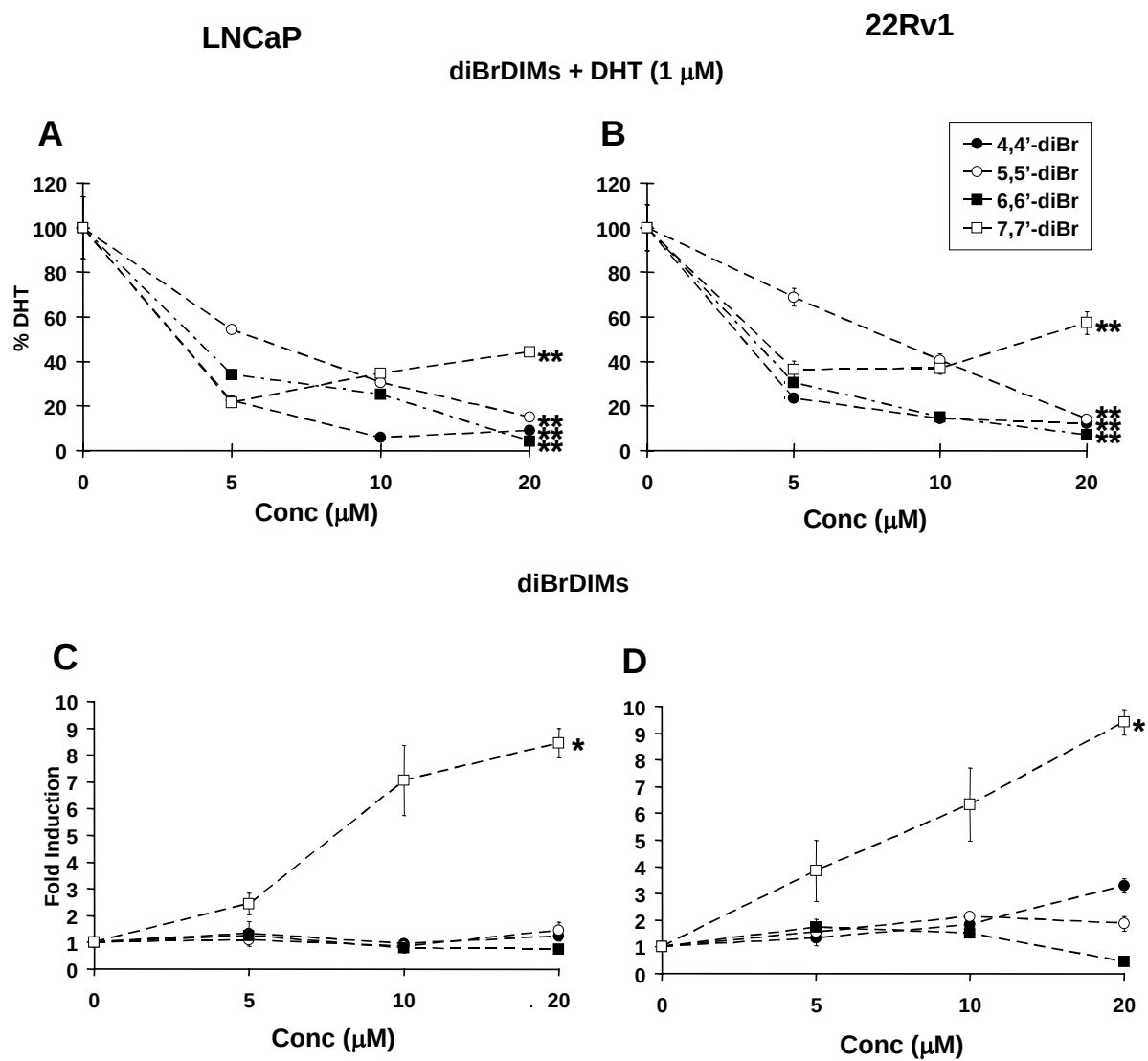


Figure 4

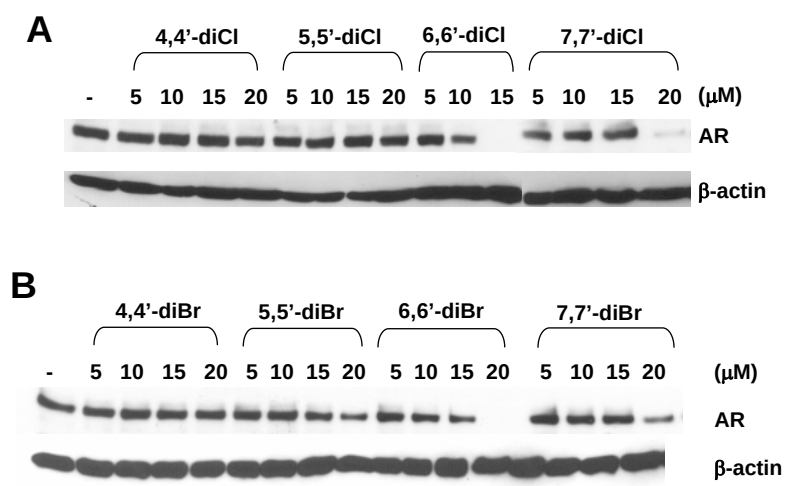


Figure 5

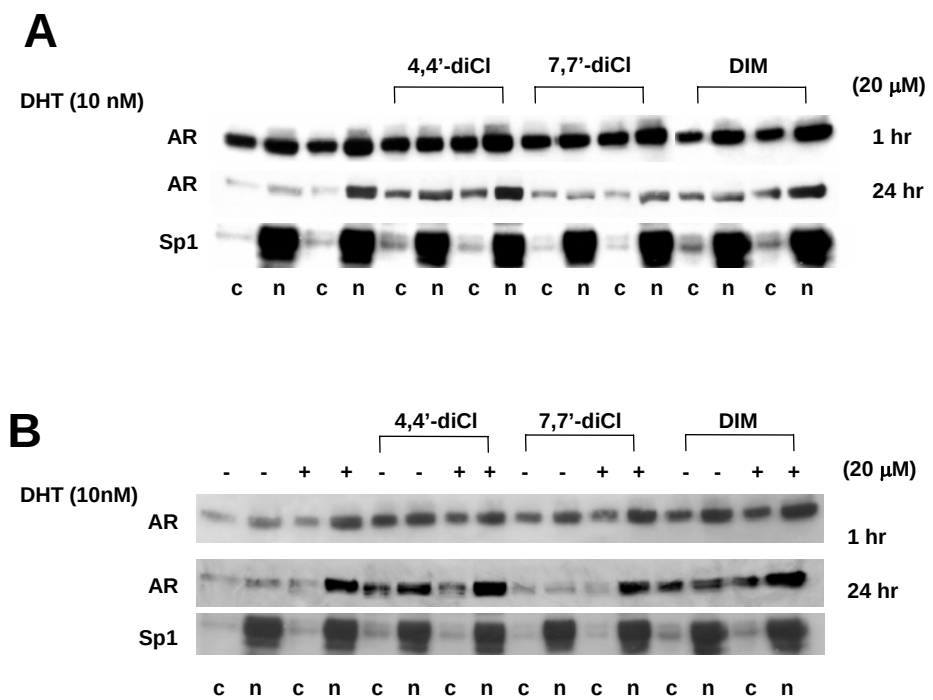


Figure 6

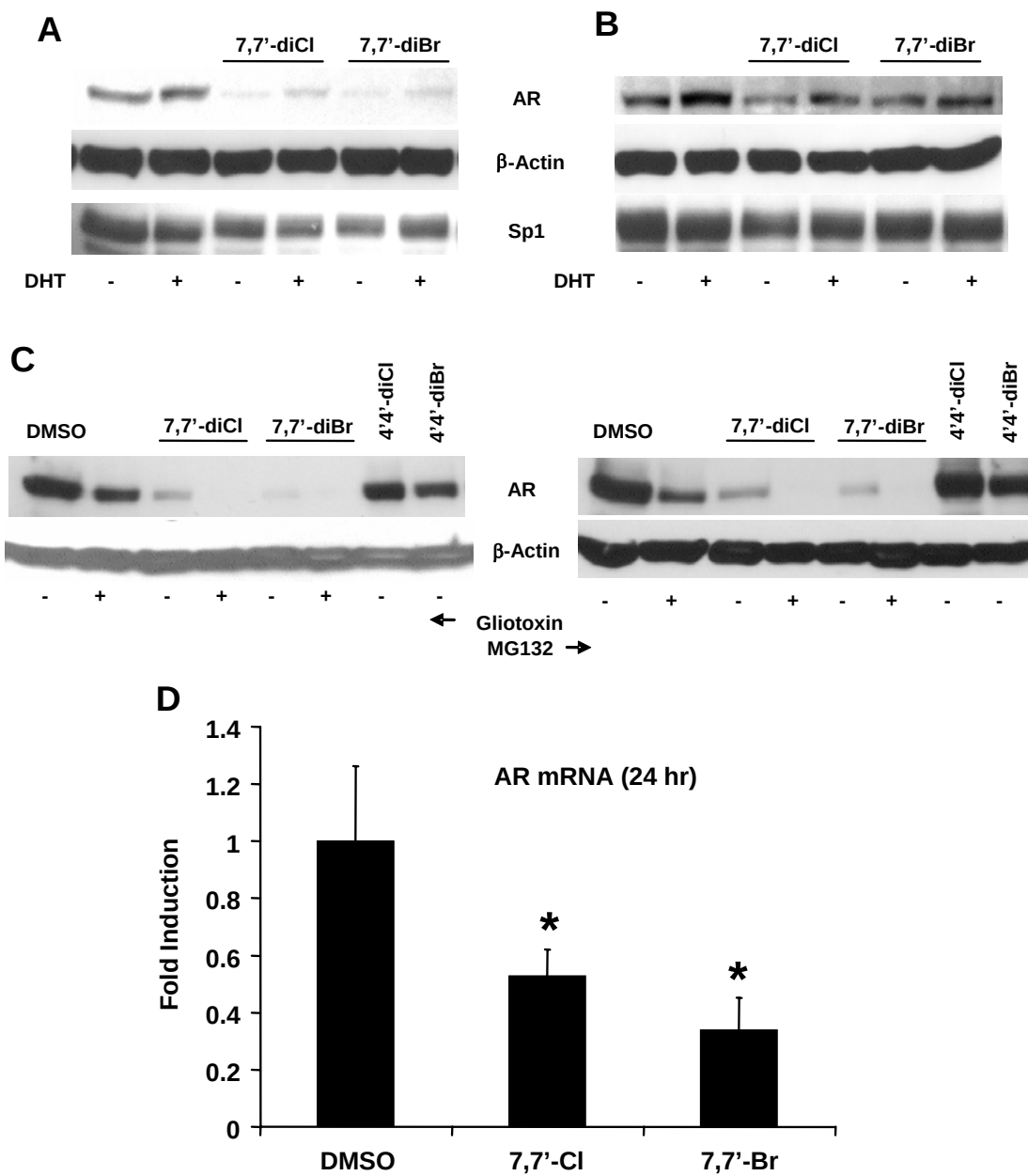


Figure 7

