

Toxic and Chemopreventive Ligands Preferentially Activate Distinct Aryl Hydrocarbon Receptor Pathways: Implications for Cancer Prevention

Steven T. Okino, Deepa Pookot, Shashwati Basak and Rajvir Dahiya

Abstract

The aryl hydrocarbon receptor (AhR) is a ligand-activated regulatory protein that controls estrogen action through two distinct pathways. In one pathway, AhR acts as a transcription factor that induces the expression of the *CYP1* family of estrogen-metabolizing genes; in the other pathway, AhR initiates the degradation of the estrogen receptor and suppresses estrogen signaling. The AhR ligand 3,3'-diindolylmethane (DIM) is a beneficial dietary constituent that prevents breast tumors in rodents and is associated with decreased breast cancer risk in humans. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is a toxic AhR ligand that is implicated in birth defects, infertility, and cancer. We analyzed MCF-7 cells to gain insight into how two AhR ligands can exert such fundamentally different health effects. We find that DIM and TCDD have differing abilities to activate the distinct AhR-controlled pathways. TCDD strongly induces AhR-dependent *CYP1* gene expression, whereas DIM is a relatively weak *CYP1* inducer. DIM strongly inhibits estrogen receptor- α expression and estrogen signaling, whereas TCDD has a notably weaker effect on these processes. Small interfering RNA knockdown of AhR confirms that the effects of DIM and TCDD are indeed AhR dependent. Our findings reveal that DIM and TCDD each elicit a unique pattern of change in pathways that control estrogen action; such patterns may determine if an AhR ligand has beneficial or adverse health effects.

There is clear evidence that estrogen is associated with breast cancer via two distinct pathways. In one pathway, estrogen metabolites act as carcinogens that damage DNA and initiate breast tumors. In the other pathway, estrogen activates the estrogen receptor (ER), which stimulates the growth of cancerous breast cells (1, 2). The aryl hydrocarbon receptor (AhR), a ligand-activated regulatory protein, controls critical steps in both of these pathways. AhR activates the expression of genes that metabolize estrogen (1, 3) and it initiates the degradation of the ER (4, 5). By controlling these pathways, AhR can influence breast cancer initiation and progression.

Epidemiologic studies show that consumption of cruciferous vegetables may provide protection against breast cancer (6). Cruciferous vegetables contain chemopreventive compounds that prevent breast tumors in mice and affect estrogen metabolism and signaling (7–10). Interestingly, these compounds also bind to and activate AhR (11–13). In this study, we compare

the AhR-dependent effects of 3,3'-diindolylmethane (DIM), a commercially available nutritional supplement associated with cruciferous vegetables, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a toxic environmental contaminant (14, 15).

We find that DIM and TCDD differ in their ability to activate the distinct AhR-controlled pathway, which implies that the biological effects of different AhR ligands may vary considerably. These findings may help to explain how DIM and TCDD can exert such fundamentally different health effects and suggest that AhR may play a role in cancer chemoprotection.

Materials and Methods

Cells and cell culture

MCF-7 cells were obtained from the American Type Culture Collection and cultured as directed. The cells were grown in phenol red-free α -MEM (Invitrogen) supplemented with 5% charcoal/dextran-treated fetal bovine serum (Hyclone) for at least 5 d before harvest. Without this specialized medium, we found that results were inconsistent due to the likely uncontrolled exposure to low levels of endogenous ER- α and AhR ligands. Cells were treated with DIM (Biomol International LP), TCDD (Wellington Laboratories, Inc.), and/or estradiol (E2; Sigma-Aldrich) as indicated.

Small interfering RNA treatment

A pool of four small interfering RNAs (siRNA) that target AhR or a pool of four nontargeting control siRNAs was purchased from Dharmacon and transfected into cells using DharmaFECT 4 (Dharmacon) as directed by the manufacturer. Cells were harvested and analyzed 72 to 96 h following transfection.

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Analysis of mRNA

Total RNA was isolated and converted to cDNA as before (16). For conventional PCR, specific mRNAs were amplified and analyzed as described previously (16). Quantitative PCR was done using Taqman primers on a 7500 Fast Real-Time System as directed by the manufacturer (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control to normalize the mRNA expression data. At least two samples isolated independently were analyzed and the results were averaged.

Analysis of protein

Protein was analyzed by Western blot analysis as previously described (17). The antibodies used to detect AhR and ER- α were purchased from Santa Cruz Biotechnology, Inc. The antibody used to detect GAPDH was purchased from Chemicon International, Inc.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) analysis was done as described previously (16).

Results

We compared the AhR-dependent effects of DIM and TCDD in the human mammary carcinoma cell line MCF-7. We analyzed DIM at levels that can be achieved in plasma following consumption of a serving of cruciferous vegetables (10 μ mol/L; ref. 18) or by using a DIM nutritional supplement (30 μ mol/L; ref. 19). TCDD was assayed at low levels (50 and 100 pmol/L) and at a maximal dose (10 nmol/L). We first assessed the expression of the AhR-regulated *CYP1* family of estrogen-metabolizing genes, *CYP1A1*, *CYP1A2*, and *CYP1B1*. *CYP1A1* and *CYP1A2* metabolize E2 to 2-hydroxyestradiol (2-OHE2), a noncarcinogenic compound (1, 3). 2-OHE2 can be further metabolized to 2-methoxyestradiol (2-MeOE2), a beneficial compound that protects against breast tumors in rodents and is being studied as a treatment for human breast cancer (20, 21). *CYP1B1* metabolizes E2 to 4-hydroxyestradiol (4-OHE2), a highly genotoxic and carcinogenic metabolite (1, 3).

We find that DIM strongly induces all *CYP1* mRNAs in a dose-dependent fashion (Table 1). Induction at 30 μ mol/L DIM was 310-, 49-, and 11-fold for *CYP1A1*, *CYP1A2*, and *CYP1B1*, respectively. Higher doses of DIM did not increase *CYP1* mRNA levels further (data not shown). A high dose of TCDD (10 nmol/L) induced all *CYP1* mRNAs to a greater ex-

tent than DIM. Lower doses of TCDD induced *CYP1* mRNAs similar to DIM. When assessing *CYP1* mRNA expression relative to GAPDH, we find that basal expression of *CYP1A1* and *CYP1A2* is very low (0.05% and 0.0014% of GAPDH), whereas *CYP1B1* expression is moderate (5% of GAPDH). Following DIM or TCDD treatment, *CYP1A1* and *CYP1B1* mRNA levels increase to moderate to high levels (4.4-91% of GAPDH), whereas *CYP1A2* expression remains low. The ratio of *CYP1B1*:*CYP1A1* mRNA (a reflection of the 4-OHE2:2-OHE2 metabolite ratio) is high under basal conditions and decreases significantly in cells exposed to DIM or TCDD. This implies that these compounds could elicit a chemoprotective effect by shifting estrogen metabolism away from the formation of a carcinogenic metabolite (4-OHE2) and toward the formation of a noncarcinogenic metabolite (2-OHE2) that can be converted into a cancer chemopreventive agent.

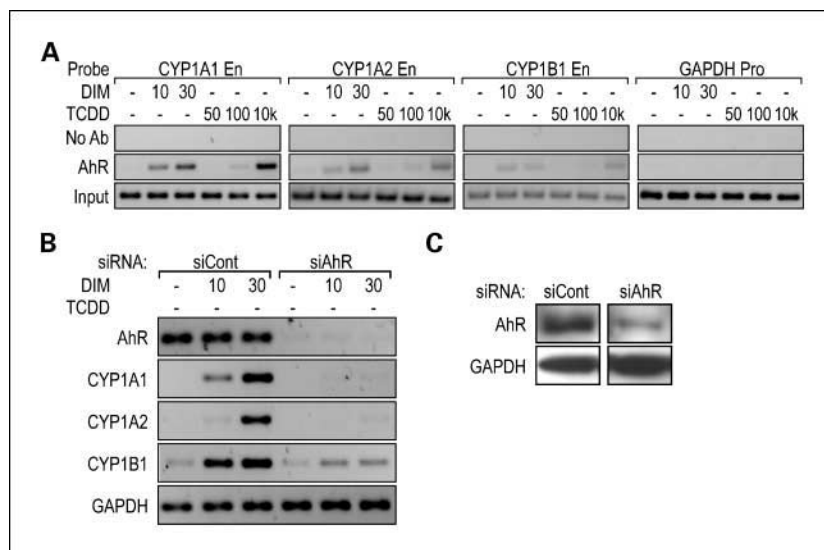
AhR activates *CYP1* gene expression by binding to the corresponding enhancer as a transcription factor (16, 22, 23). We analyzed the transcription factor activity of AhR by measuring its interaction with the *CYP1* enhancers by ChIP (Fig. 1A). We observe dose-dependent, DIM-inducible AhR binding on all *CYP1* enhancers. The extent of AhR enhancer binding induced by 30 μ mol/L DIM is similar to that induced by a high dose of TCDD. However, because DIM is a relatively weak *CYP1* mRNA inducer (Table 1), we infer that it does not activate the ability of AhR to alter gene expression as well as TCDD does. Our finding that cells treated with DIM exhibit markedly more AhR enhancer binding than cells treated with low doses of TCDD (which induce a similar level of *CYP1* expression) also implies that the DIM-AhR complex, in comparison with the TCDD-AhR complex, is a relatively weak activator of *CYP1* gene expression. These findings reveal that DIM and TCDD have notably different abilities to activate the transcription factor function of AhR. Hestermann and Brown (24) have used ChIP to monitor recruitment of AHR, coactivators, and Pol II to, and acetylation of histones on, the *CYP1A1* promoter *in vivo*. Their results have shown that an AHR agonist causes cycles of receptor and cofactor recruitment leading to gene transcription. In contrast, DIM, the selective AHR modulator, recruited a subset of cofactors but failed to cause histone acetylation or effective polymerase recruitment. These results suggest a mechanism for the chemopreventive activity of DIM and related compounds (24).

Table 1. Induction of *CYP1* mRNAs by DIM and TCDD

	Dose	Fold induction			Expression level (% GAPDH)			Ratio 1B1/1A1
		CYP1A1	CYP1A2	CYP1B1	CYP1A1	CYP1A2	CYP1B1	
Control	—	1	1	1	0.05	0.0014	5.0	99
DIM	10 μ mol/L	89	21	4.1	4.4	0.030	20.3	4.6
DIM	30 μ mol/L	310	49	11.0	15.5	0.071	54.4	3.5
TCDD	50 pmol/L	182	71	6.3	9.1	0.102	31.1	3.4
TCDD	100 pmol/L	544	154	10.1	27.2	0.219	50.2	1.8
TCDD	10 nmol/L	1,819	1,037	16.9	91	1.48	84	0.9

NOTE: RNA isolated from MCF-7 cells treated for 18 h with the indicated doses of DIM or TCDD was analyzed by real-time PCR to assess the level of *CYP1A1*, *CYP1A2*, *CYP1B1*, and GAPDH RNA. The fold induction was calculated as the ratio of induced *CYP1* RNA to basal *CYP1* RNA after GAPDH normalization. The expression level was calculated as (*CYP1* RNA level/GAPDH RNA level) \times 100. The ratio 1B1/1A1 was calculated as (*CYP1B1* RNA level/*CYP1A1* RNA level) \times 100. The results represent an average of two independent samples.

Fig. 1. DIM and TCDD use AhR to induce the CYP1 genes. *A*, AhR binding on the CYP1 enhancers and GAPDH promoter was assessed in cells treated with the indicated doses of DIM or TCDD for 1 h by ChIP. *B*, the effect of AhR knockdown on CYP1 induction was assessed in cells treated with a control siRNA (*siCont*) or a siRNA that recognizes AhR (*siAhR*) for 72 h followed by treatment with the indicated doses of DIM for 18 h. RNA was isolated and analyzed by reverse transcription-PCR. *C*, siRNA knockdown of AhR was assessed in MCF-7 cells treated with *siCont* or *siAhR* for 96 h by Western blot.



We then suppressed AhR expression using siRNA methodology. Transfection of a siRNA that targets AhR decreases AhR mRNA expression by ~90% compared with cells treated with a control siRNA (Fig. 1B). The level of AhR protein is also significantly decreased (Fig. 1C). Not surprisingly, in cells in which AhR is depleted, induction of the CYP1 mRNAs is markedly suppressed (Fig. 1B). These findings confirm that CYP1 induction by DIM is AhR dependent.

AhR activation also initiates ER- α degradation and suppresses E2 induction of estrogen-responsive genes (4, 5). AhR can thus exert significant antiestrogenic activity. Because breast cancer is strongly associated with estrogen exposure (1), such antiestrogenic activity may act to prevent breast carcinogenesis. We find that cells treated with DIM for 3 hours exhibit a large, dose-dependent depletion of ER- α . A high dose of TCDD also strongly depletes ER- α at this time point; lower TCDD doses result in moderate ER- α depletion (Fig. 2A). Cycloheximide, a protein synthesis inhibitor, does not affect the ability of DIM to deplete ER- α (Fig. 2B), suggesting that the activity of DIM toward ER- α is a primary response. Interestingly, after 18 hours of treatment, ER- α expression is much lower in the DIM-exposed cells than in the cells exposed to TCDD (Fig. 2C). This shows that DIM elicits a sustained depletion of ER- α , whereas the effects of TCDD are more transient. These findings also reveal that the ability of DIM to suppress ER- α expression is greater than the ability of TCDD and implies that DIM should exhibit stronger antiestrogenic activity.

We next assessed the effects of DIM and TCDD on the expression of estrogen-responsive genes. We find that in cells transfected with a control siRNA, the *SGKL*, *RET*, *NPY1R*, and *PGR* genes are all strongly induced by E2 following 18 hours of hormone treatment. However, in cells that have been co-treated with DIM or TCDD, E2 induction of these genes is greatly suppressed (Fig. 3). These results are highly consistent with results analyzing mock-transfected cells and nontransfected cells (data not shown) and indicate that DIM and TCDD both exert strong antiestrogenic activity. We also find that DIM suppresses E2 induction of all estrogen-responsive genes to a greater extent than TCDD does. This implies, as predicted above, that the DIM-AhR complex has stronger

antiestrogenic activity than the TCDD-AhR complex. These findings again reveal that DIM and TCDD differ in their ability to activate specific AhR-controlled pathways. The antiestrogenic effects of DIM may also be AhR independent based on the broad range of effects of this compound in breast and other cancer cell lines. Therefore, it may be possible that the inhibitory AhR-ER- α cross-talk by DIM and TCDD is similar but the difference between them could be the AhR-independent effects of DIM.

We also analyzed cells in which AhR is depleted by transfection with *siAhR* (Fig. 3). We find that AhR depletion does not affect E2 induction of the estrogen-responsive genes.

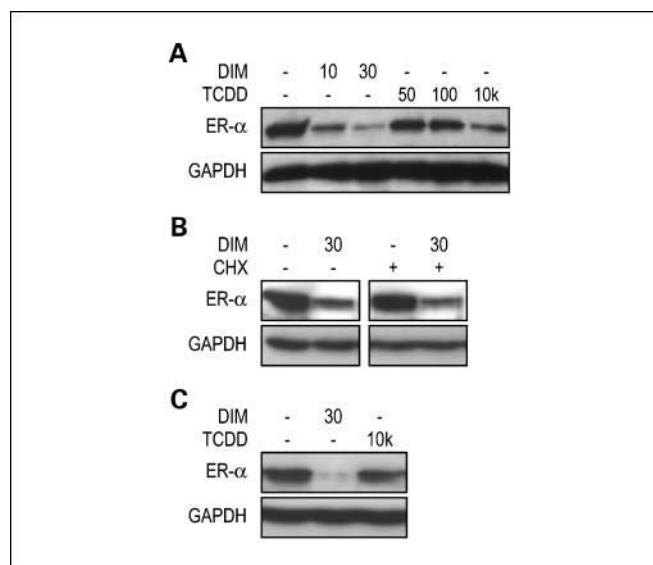


Fig. 2. DIM and TCDD suppress ER- α expression. *A*, ER- α expression was assessed in cells treated with the indicated doses of DIM or TCDD for 3 h by Western analysis. *B*, ER- α expression was assessed in control cells or in cells treated with DIM and/or cycloheximide (CHX) for 3 h by Western analysis. *C*, ER- α expression was assessed in cells treated with the indicated doses of DIM or TCDD for 18 h by Western analysis.

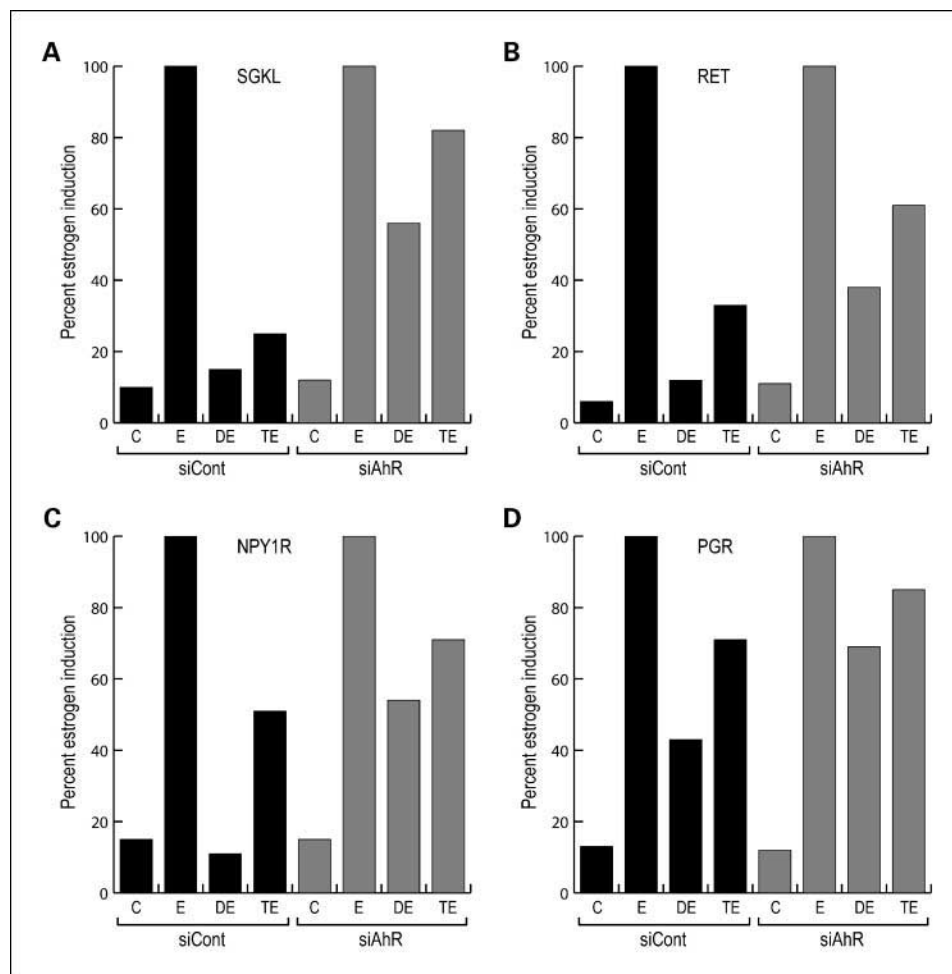


Fig. 3. DIM and TCDD use AhR to suppress estrogen signaling. MCF-7 cells were treated with siCont or siAhR for 72 h. The cells were then treated for 18 h with vehicle (C), 10 nmol/L E2 (E), 30 μ mol/L DIM and 10 nmol/L E2 (DE), or 10 nmol/L TCDD and 10 nmol/L E2 (TE). RNA was isolated and the level of SGKL, RET, NPY1R, and PGR was determined by real-time PCR using GAPDH for normalization. The results are expressed as the percent estrogen induction, which was calculated as (mRNA level in treatment group/mRNA level in cells treated with E2 only) \times 100. The results represent an average of two independent samples.

However, it strongly diminishes the ability of DIM and TCDD to suppress E2-induced gene expression. This confirms that AhR plays a key role in facilitating the antiestrogenic effects of DIM and TCDD. Because breast tumor growth is often resultant of estrogen action (1), such AhR-dependent, antiestrogenic activity may act to suppress breast cancer.

Discussion

AhR ligands are a structurally diverse group of natural and synthetic compounds that elicit a broad range of biological effects (13, 14, 25). Here, we analyze two AhR ligands, DIM and TCDD, which have opposing effects on human and animal health. DIM is a beneficial dietary constituent that suppresses breast tumors in rodents (9, 10) and may decrease breast cancer risk in humans (6). TCDD is a highly toxic, man-made environmental contaminant that is implicated in birth defects, infertility, and cancer (14, 15, 26). We find that DIM and TCDD differ in their ability to activate the two distinct AhR-controlled pathways: transcriptional activation and hormone receptor degradation. TCDD is better than DIM at activating *CYP1* expression. DIM is better than TCDD at suppressing ER- α expression and estrogen signaling. This finding, that AhR ligands can preferentially activate the distinct AhR-controlled pathways, implies that the biological

effects of different AhR ligands may vary considerably. This may help to explain how the two studied AhR ligands can exert such fundamentally different health effects.

In human breast cells, AhR controls the effects of estrogen by regulating its metabolism and signaling. Because breast cancer is highly associated with estrogen action (1), AhR ligands may thus influence mammary carcinogenesis. Various reports have shown relatively nontoxic AhR agonists (i.e., alternate substituted alkyl polychlorinated dibenzofurans and diindolylmethane analogues), which inhibit growth of 7,12-dimethylbenz(a)anthracene-induced mammary tumor in female Sprague-Dawley rats (27–30); moreover, these compounds also exhibit antiestrogenic activity in the rodent uterus (28, 30, 31). Indeed, there is strong evidence that DIM prevents breast tumors in animals and humans (6, 9, 10). Surprisingly, studies also suggest that TCDD suppresses spontaneous and chemically induced breast cancer in rodents (32, 33). These findings imply that AhR protects against breast cancer. AhR elicits its effects using two separate pathways: transcriptional activation and hormone receptor degradation. We find that activation of both of these pathways produces effects that might offer breast cancer chemoprotection. By inducing *CYP1* transcription, AhR alters the expression of estrogen-metabolizing genes such that the formation of noncarcinogenic and chemopreventive E2 metabolites would be favored over carcinogenic metabolites. By degrading ER- α , AhR inhibits

estrogen signaling, which can suppress the progression of estrogen-dependent breast tumors. AhR may thus play an important role in breast cancer chemoprevention.

AhR might also be involved in prostate cancer chemoprevention. In its initial stages, prostate tumors are dependent on androgens for growth. In human prostate cells, AhR activation initiates androgen receptor (AR) degradation and suppresses the induction of an androgen-responsive reporter gene (5); such antiandrogenic effects might inhibit prostate cancer progression. There is evidence that DIM can suppress androgen action. Human prostate cells exposed to DIM exhibit decreased AR expression and suppressed androgen responsiveness (18, 34). Cruciferous vegetable consumption is also associated with decreased prostate cancer risk (6, 35). These findings imply that AhR may be involved in the chemoprevention of human cancers at multiple sites. AhR-regulated CYP1 family of estrogen-metabolizing genes includes CYP1A1, CYP1A2, and CYP1B1. CYP1A1 and CYP1A2 metabolize E2 to 2-OHE2, which is a noncarcinogenic compound. 2-OHE2 can be further metabolized to 2-MeOE2, which is a beneficial compound that protects against breast tumors in rodents and is being studied as a treatment for human breast cancer. CYP1B1 metabolizes E2 to 4-OHE2, a highly genotoxic and carcinogenic metabolite. We found that DIM strongly induces all CYP1 mRNAs in a dose-dependent fashion. Data are compared with the basal levels. The ratio of CYP1B1:CYP1A1 mRNA (a reflection of the 4-OHE2:2-OHE2 metabolite ratio) is high under basal conditions (1B1/1A1 = 99) and decreases significantly in cells exposed to DIM (3.5) or TCDD (0.9). This implies that these compounds could elicit a chemoprotective effect by shifting estrogen metabolism away from the formation of a carcinogenic metabolite (4-OHE2) and toward the formation of a noncarcinogenic metabolite (2-OHE2) that can be converted into a cancer chemopreventive agent.

AhR is a relatively promiscuous receptor that binds a broad range of natural and synthetic compounds (13, 25). It is thus expected that some AhR ligands might act similar to DIM and offer protection against specific cancers. Genistein, an AhR

ligand that is abundant in soy, has striking similarities to DIM in that it is a weak CYP1 inducer (36) that suppresses ER- α and AR expression in human breast and prostate cells, respectively (37, 38). In addition, genistein consumption prevents breast and prostate tumors in rodents (39) and is associated with decreased risk of these cancers in humans (40). In preliminary studies analyzing MCF-7 cells, we find that genistein is less effective than DIM at inducing CYP1 transcription and equivalent to DIM at suppressing ER- α expression (data not shown). It will be interesting to determine if these properties are associated with cancer chemoprevention. Similarly, it will also be important to determine the functional properties that distinguish between toxic and beneficial AhR ligands.

AhR was initially thought of as an inducible transcription factor that controls the expression of enzymes that metabolize potentially dangerous xenobiotic chemicals (26). Later studies show that the physiologic role of AhR is significantly more diverse and includes a possible role in liver development (25). The effects of AhR on hormonal systems were identified because "endocrine-disrupting" AhR ligands, such as TCDD, adversely affect animal reproductive systems (14, 15). Here, we show that AhR may elicit beneficial effects on hormonal systems by preventing the progression of certain cancers. Interestingly, beneficial AhR ligands are natural dietary compounds. Thus, it seems that AhR may play a role in mediating the beneficial effects of a healthy diet.

It is important to note that the concept of selective AhR modulators to treat hormone-dependent cancers was first proposed by Dr. Stephen Safe and colleagues (41). In particular, he believed that AhR ligands can be used to modulate hormonal pathways without exhibiting toxicity. Our findings support his views and indicate that AhR mediates processes that can decrease breast cancer initiation and progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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