ABSTRACT

The polycyclic aromatic hydrocarbon 6-methyl-1,3,8-trichlorodibenzo-furan (MCDF) is related to the industrial byproduct dioxin and is a weak agonist and partial antagonist at the aryl hydrocarbon receptor (AhR). Tamoxifen is used for the treatment and prevention of breast cancer and interferes with the interaction of estrogen with estrogen receptor α (ER). The combination of MCDF and tamoxifen lowered the effective dose of both drugs required to inhibit 7,12-dimethylbenz(a)anthracene-induced mammary tumor growth in rats and protected against the estrogenic effects of tamoxifen on the uterus in rats (A. McDougal et al., Cancer Res 2001;61:3902–7), pointing to the potential use of MCDF in breast cancer treatment. Potential AhR-ER cross-talk is evidenced by the antiestrogenic activity of MCDF and the degradative effect of MCDF on ER protein levels. Our studies confirmed that MCDF degraded the ER. MCDF displayed antiestrogenic activity at higher concentrations in MCF-7 human breast cancer cells, but MCDF alone (10−9 m) stimulated the growth of MCF-7 cells. MCDF also activated an estrogen response element (ERE)-luciferase reporter and increased mRNA levels of the estrogen-responsive gene transforming growth factor (TGF)-α. The estrogenic effects of MCDF are ER dependent because they were blocked by the pure antiestrogen ICI 182,780. MCDF induced ER-coactivator interaction in glutathione S-transferase pull-down assays and the formation of an ER-ERE complex in gel mobility shift assays, further indicating that the estrogenic actions of MCDF are mediated by the ER. In addition, knockdown of the AhR with small interfering RNA did not affect MCDF-induced ERE-luciferase activity. Overall, these data support the conclusion that MCDF is a partial agonist at the ER. This study provides the first evidence for the direct interaction of the ER with MCDF and challenges the view that MCDF is simply an AhR-specific ligand.

INTRODUCTION

2,3,7,8-Tetrachlorodibenz(o-p)-dioxin (TCDD) is a member of a family of polycyclic aromatic hydrocarbons. TCDD is a byproduct of industrial processes and the combustion of organic materials (1) and is considered the most toxic member of this family. 6-Methyl-1,3,8-trichlorodibenzo-furan (MCDF) is a related compound that is relatively nontoxic (Fig. 1).

The effects of TCDD and MCDF are mediated through the aryl hydrocarbon receptor (AhR). The AhR is a ligand-activated nuclear transcription factor that has no identified endogenous ligand (2). When a ligand such as TCDD or MCDF binds to the AhR, the AhR forms a heterodimer with AhR nuclear translocator protein (Arnt), and this transcription complex interacts with dioxin or xenobiotic response elements in the 5′ regulatory region of Ah-responsive genes. MCDF is a weak agonist and partial antagonist at the AhR (3). The overall mechanism of action is similar to that observed for other nuclear hormone receptors, such as estrogen receptor α (ER).

Current treatment strategies of ER-positive breast cancer in the clinic involve the use of selective estrogen receptor modulators and pure antiestrogens. Tamoxifen is a selective estrogen receptor modulator used for the treatment and prevention of breast cancer (4). Tamoxifen acts as an antiestrogen in the breast but acts as an estrogen in bone, liver, and uterus (5). This estrogen-like activity in the uterus results in an increased incidence of endometrial cancer in women over age 50 years (6). Tamoxifen interferes with the binding of estrogen to the ER (7), although the molecular mechanism of the antiestrogenicity of tamoxifen is more complex. ICI 182,780 is a pure antiestrogen that possesses no estrogen-like effects in the uterus, and ICI 182,780 treatment results in the degradation of the ER (8). ICI 182,780 is currently available as a second-line therapy after the development of tamoxifen resistance (9, 10).

MCDF is being investigated as an agent to treat breast cancer because of its antiestrogenic activity (11). Studies in vitro demonstrate that MCDF inhibits 17β-estradiol (E2)-induced cell proliferation and E2-induced chloramphenicol acetyltransferase (CAT) activity from a vimentin-CAT reporter in MCF-7 breast cancer cells (12, 13). Studies in vivo in rats indicate that MCDF prevents E2-induced increases in ER and progesterone receptor in the uterus and liver, as well as uterine wet weight increases (14). MCDF also inhibits tumor growth in the 7,12-dimethylbenz(a)anthracene-induced rat mammary tumor model (15).

Cross-talk between the AhR and the ER has been observed in a variety of systems. For example, TCDD and MCDF degrade ER protein in MCF-7 human breast cancer cells in a proteasome-dependent manner (12, 16, 17), and the AhR is required for this degradation (16). Further interactions are observed in vivo. In the 7,12-dimethylbenz(a)anthracene-initiated rat mammary tumor model, 100 μg/kg tamoxifen and 50 μg/kg MCDF inhibited tumor growth (17). When tamoxifen and MCDF were combined using doses that were independently inactive, tumor growth was inhibited. MCDF also inhibited tamoxifen-induced uterine responses, such as progesterone receptor binding and peroxidase activity, without altering the beneficial effects of tamoxifen. The use of MCDF in combination with tamoxifen may lower the effective dose of both drugs, thereby protecting against the estrogenic effects of tamoxifen in the uterus.

Many studies have focused on the interactions between MCDF and the AhR, but few have analyzed the interaction between MCDF and the ER suggested in previous studies (16, 17). To investigate MCDF-ER interactions, we have used three different assays to evaluate the estrogenic or antiestrogenic actions of MCDF: (a) growth assays in MCF-7 cells; (b) estrogen response element (ERE)-luciferase reporter transfections in ER-positive MCF-7 cells and ER-negative T47D:C4:2 cells; and (c) analysis of the expression of an endogenous E2-responsive gene, transforming growth factor (TGF)-α, in MDA-MB-231 cells stably transfected with ER cDNA. These model systems were used because their responses to estrogens and antiestrogens are well characterized (18–21). Each of these assays indicated that MCDF stimulated cell growth, activated an ERE-luciferase re-
porter, and induced TGF-α mRNA in an ER-dependent manner. A recent report by Ohtake et al. (22) showed that an AhR ligand [3-methylcholanthrene (3MC)] activated an ERE-luciferase reporter by forming a complex between the ER, AhR, Arnt, and 3MC. In this study, knockdown of the AhR with small interfering RNA (siRNA) did not affect MCDF-induced estrogenic activity, suggesting that the mechanism of MCDF action in breast cancer cells involves a direct activation of the ER by MCDF.

**MATERIALS AND METHODS**

**Cell Culture and Reagents.** MCF-7 human breast cancer cells (ER positive) were obtained from American Type Culture Collection and maintained in RPMI 1640 containing 10% fetal bovine serum, 2 mM t-glutamine, 0.1 mM nonessential amino acids, 100 units/ml penicillin, 100 μg/ml streptomycin, and 6 mg/ml insulin. MDA-MB-231 human breast cancer cells stably transfected with the wild-type (wt) ER (S30 cells, referred to as wt-ER; Ref. 23) were grown in phenol red-free MEM supplemented with 5% dextran-coated charcoal-treated calf serum, 0.5 mg/ml Gentinovic (Invitrogen Life Technologies, Inc., Carlsbad, CA), and concentrations of glutamine, nonessential amino acids, penicillin, streptomycin, and insulin described above. T47D-C4:2 human breast cancer cells (Ref. 24; ER negative) were propagated in phenol red-free RPMI 1640 containing 10% dextran-coated charcoal-treated fetal bovine serum as well as the additions described for the MCF-7 cells.

MCDF (synthesized by Dr. Stephen Safe (25)) was dissolved in DMSO and stored at room temperature. 4-Hydroxytamoxifen and E2 were dissolved in ethanol and purchased from Sigma-Aldrich Co. (St. Louis, MO). ICI 182,780 was dissolved in ethanol and obtained from AstraZeneca (Macclesfield, United Kingdom). All drugs except MCDF were stored at −20°C.

**Protein Isolation and Western Blots.** MCF-7 cells were transferred into estrogen-free media (phenol red-free RPMI 1640 with 10% dextran-coated charcoal-treated fetal bovine serum) for 4 days before drug treatments. The cells were treated for 24 h with compounds as indicated. Protein was harvested as described previously (21).

**Cell Growth Assays.** MCF-7 cells were transfected with 1 μg of the pERE-luciferase plasmid alone or 1 μg of the ER expression plasmid pSG5-HEGO (provided by P. Chambon) along with 1 μg of the pERE-luciferase reporter plasmid. To normalize for transfection efficiency, 0.2 μg of the pcMVβ plasmid was cotransfected. Cells (5 × 10^5) were electroporated in a 0.4-cm cuvette (Bio-Rad Laboratories) at a voltage of 0.320 kV and a high capacitance of 950 μF in a Bio-Rad Gene Pulser II (Bio-Rad Laboratories) in serum-free media. The cells were transferred to 12-well plates and incubated overnight. The next day, the cells were treated with the appropriate compound in estrogen-free media for 24 h. Cell lysates were prepared as described previously (21). Data are reported as relative light units (the luciferase reading divided by the β-galactosidase reading).

**Northern Blots.** The wt-ER cells were treated for 24 h with the appropriate compound. Total RNA was isolated using Trizol reagent (Invitrogen Life Technologies, Inc.) according to the manufacturer’s instructions. Twenty μg of RNA were loaded per lane in a 1% agarose/0.66 M formaldehyde gel. The Northern blots for TGF-α and β-actin were performed as described previously (21).

**Glutathione S-Transferase (GST) Pull-Down Assays.** GST pull-down assays were performed as described previously (28). ^35S-labeled AIB1 was made from pcDNA-3.1-AB1 (kindly provided by P. Meltzer, NIH, Bethesda, MD), using an in vitro transcription-translation-coupled translation system (Promega, Madison, WI).

**Gene Mobility Shift Assays.** The human AhR expression plasmid AhR-pcDNA was obtained from Dr. Stephen Safe. The human Arnt expression plasmid Arnt-plSPORT (PL87) was obtained from Dr. Chris Bradfield (University of Wisconsin). The proteins were generated using the TNT Coupled Reticulocyte Lysate System (Promega), a coupled transcription/translation system.

For the gel shift assay, the ERE probe (ERE1, 5′-GAT-CTC-TTT-GAT-CAG-CGC-ACC-TCA-GGT-CAC-AGT-GAC-CTG-ATC-AAA-GA-3′; ERE2, 5′-GAT-CCA-AAG-TCA-GGT-CAC-AGT-GAC-CTG-ATC-AAA-GA-3′) or dioxin response element (DRE) probe (DRE1, 5′-GAT-CTG-CTT-CTT-ATG-GAA-GAG-GTC-3′; DRE2, 5′-GAT-CGC-GAG-TGG-CGT-GAG-AAG-GAC-3′) was labeled by combining 250 ng of the annealed ERE or DRE oligonucleotide; 2 μl of 10X NEB buffer 3 (New England BioLabs, Beverly, MA); 1 μl each of a 5 mM stock of dATP, dGTP, and dTTP; 8 μl of water; 1 μl of Klenow; and 5 μCi of [35P]UTP (Amersham Biosciences). The labeled probe was isolated using Microspin columns (Amersham Biosciences).

A typical gel shift reaction consisted of 200 μg of BSA, 3 μl of the appropriate translated protein(s) or 20 ng of purified ER (PanVera, Madison, WI), and buffer (20 mM HEPES (pH 7.9), 20% glycerol, and 100 mM KCl) to a total of 14.65 μl. ICI 182,780 was added for 10 min at 37°C before adding other compounds or antibodies (G20 for ER, sc-8088 for AhR, and sc-8076 for Arnt; Santa Cruz Biotechnology). The reaction was then incubated for 10 min at 37°C. Then, 1.5 μl of a 1 μg/μl stock of poly(deoxyinosinic-deoxythymidylic acid), 1.4 μl of 0.1 M MgCl2, and 1.5 μl of the labeled ERE were added before an incubation at 37°C for 10 min. The samples were run on a 5% polyacrylamide gel, and the gel was dried and exposed to film overnight.

**Transient Transfection Studies with siRNAs.** siRNA duplexes were prepared by Dharmacon Research (Lafayette, CO) and targeted coding regions of the AhR (nucleotide positions 1416 to 1434) and GL2 (luciferase) (nucleotide positions 153 to 171). Scrubbed inhibitory RNA (iRNA) (iSC) was derived from a message transcribed from the chloroplast genome of Euglena gracilis (GenBank accession number X78010: position 24750–24768). The siRNA duplexes used in this study are indicated below: (a) iGL2, 5′-CGG-CGG-AAG-AAU-UCG-ATT and TT-GGA-GUC-GGC-UAA-UAG-AUC-GC-U-5′; (b) iSC, 5′-CG-GCU-UUC-GUA-GGA-GGA-UCC-GTT and TT-GCG-GCG-AAA-CAU-CCU-AAG-C-5′; and (c) iAbR, 5′-UAC-UUC-CAC-CUC-AUG-UGG-ATT and TT-AUG-AAG-GAG-GAG-UCA-ACC-G-5′.

Cells were cultured in 6-well plates in 2 ml of DMEM/Ham’s F-12 medium supplemented with 2.5% stripped fetal bovine serum. After 16–20 h, siRNA duplexes and reporter gene constructs (500 ng/well) were transfected using Oligofectamine reagent (Invitrogen Life Technologies, Inc.). siRNA duplexes were transfected in each well to give a final concentration of 100 nM. Twenty-four h after transfection, cells were treated with DMSO, 10 nM E2, or 2 μM MCDF for 24–36 h. Cells were then harvested, and luciferase activity (relative to β-galactosidase activity) was determined.
ER mRNA in wt-ER cells, whereas MCDF and ICI 182,780 did not change ER mRNA levels (data not shown), indicating that MCDF and ICI 182,780 have similar effects on ER mRNA levels.

**Growth Assays in MCF-7 Cells.** Cell growth assays were performed to determine the effect of MCDF on the growth of MCF-7 cells. MCF-7 cells were treated with various compounds for 6 days, and cellular DNA was quantitated as a measure of cell number. E2 concentrations of $10^{-13}$ to $10^{-8}$ M caused a concentration-dependent increase in MCF-7 cell growth, compared with the ethanol/DMSO control, with a plateau at $10^{-10}$ M (Fig. 3A). 4-Hydroxytamoxifen or ICI 182,780 treatment at concentrations of $10^{-11}$ to $10^{-6}$ M had no effect on MCF-7 cell growth. Treatment of the cells with $10^{-11}$ to $10^{-8}$ M MCDF had no effect on cell growth, whereas $10^{-7}$ M MCDF
slightly increased growth, and 10⁻⁶ M MCDF significantly increased cell growth to a level equal to half of the maximal E₂ response. When 10⁻⁶ M MCDF and a range of E₂ concentrations were combined, a level of cell growth equivalent to a 10⁻⁶ M concentration of MCDF alone was achieved, indicating that MCDF had partial agonist activity.

An additional growth assay was performed in MCF-7 cells to further investigate the antiestrogenic activity of MCDF. As shown in the previous experiment, treatment of MCF-7 cells with MCDF alone showed a stimulation of cell growth at 10⁻⁷ and 10⁻⁶ M MCDF (Fig. 3B). The E₂ curve was also reproduced, with E₂ increasing MCF-7 cell growth. When E₂ (10⁻¹³ to 10⁻⁸ M) was combined with 10⁻⁶ M MCDF, the growth response curve was the same as that of E₂ alone. Treatment with 10⁻⁷ M MCDF in combination with 10⁻¹² or 10⁻¹³ M E₂ was similar to treatments with 10⁻⁶ M MCDF alone, whereas the combination of 10⁻⁷ M MCDF and higher concentrations of E₂ followed the E₂ alone curve. As shown previously (Fig. 3A), the growth curve of E₂ in combination with 10⁻⁶ M MCDF was similar to that of 10⁻⁶ M MCDF alone. Therefore, high concentrations of MCDF (10⁻⁶ M) can partially inhibit the E₂-induced growth stimulation of MCF-7 cells over a wide range of E₂ concentrations (10⁻¹¹ to 10⁻⁸ M).

to determine whether the stimulatory effect of MCDF is ER mediated, growth assays were performed in MCF-7 cells with E₂ or MCDF in combination with ICI 182,780 (Fig. 3C). ICI 182,780 inhibited E₂-induced cell growth. Interestingly, ICI 182,780 also completely abolished MCDF-stimulated cell growth. These results suggest that the MCDF-induced growth responses observed in MCF-7 cells are ER mediated.

**ERE-Luciferase Reporter Assays in MCF-7 and T47D:C4:2 Cells.** Because MCDF had effects on ER levels and the growth of ER-positive breast cancer cells, functional assays involving potential ER targets were used. The first assay involved testing whether MCDF could activate an ERE-luciferase reporter in T47D:C4:2 cells. T47D:C4:2 cells are ER-negative cells that were derived from ER-positive T47D cells grown in estrogen-deprived media (24). These cells were transiently transfected with a pERE-luciferase reporter plasmid (27) and a β-galactosidase plasmid (pCMVβ) to normalize for transfection efficiency. The cells were treated with various compounds for 24 h, and the resulting luciferase activity was measured. When the pERE-luciferase reporter alone was transfected (Fig. 4A, □), minimal luciferase activity was detected in all conditions tested. In contrast, when the pERE-luciferase reporter and wt-ER (pSG5-HEGO; Fig. 4A, ■) were cotransfected, E₂ treatment resulted in an 8-fold induction of luciferase activity, whereas no induction occurred with ICI 182,780 treatment. In addition, ICI 182,780 completely inhibited the E₂-induced effect. No activation of luciferase activity was observed with 0.01 μM MCDF, whereas 0.1 μM MCDF induced luciferase activity by 2-fold, and 1 μM MCDF induced luciferase activity by 7-fold. When E₂ was used in combination with 0.01 or 0.1 μM MCDF, a level of activation similar to that of E₂ alone was observed. The combination of E₂ + 1 μM MCDF yielded a result similar to that of 1 μM MCDF alone. ICI 182,780 inhibited the MCDF-induced increase in luciferase activity at all concentrations of MCDF.

The ERE-luciferase reporter assay results in MCF-7 cells (Fig. 4B) were similar to those observed in T47D:C4:2 cells transfected with the ER. E₂ significantly induced luciferase activity, and MCDF also induced ERE luciferase activity in a concentration-dependent manner. In addition, ICI 182,780 was able to block the E₂- and MCDF-induced effects. These data indicate that MCDF up-regulates ERE-luciferase activity through the ER in breast cancer cells.

**Analysis of TGF-α mRNA Levels.** A second functional ER assay involves analysis of the endogenous E₂-responsive gene TGF-α in wt-ER cells, which are MDA-MB-231 cells stably transfected with the wt-ER (23). E₂ treatment resulted in an induction of TGF-α mRNA, compared with the control (Fig. 5A). A 0.01 or 0.1 μM concentration of MCDF did not result in an increase in TGF-α mRNA. However, higher concentrations of MCDF (1 and 10 μM) resulted in a significant increase in TGF-α mRNA levels, and TGF-α mRNA levels induced by 10 μM MCDF were similar to those induced by E₂. Treatment of wt-ER cells with E₂ + MCDF resulted in TGF-α levels similar to E₂ alone, indicating that MCDF does not block this particular E₂ effect (data not shown).

Cotreatment of ICI 182,780 + MCDF was performed to determine whether the induction of TGF-α mRNA occurred through an ER-mediated pathway (Fig. 5B). ICI 182,780 inhibited the MCDF-mediated induction of TGF-α mRNA, even at the highest concentration of MCDF, indicating that the ER is required for the MCDF-induced increase in TGF-α mRNA levels.

**GST Pull-Down Assays.** The multiple assays presented here indicated that MCDF is a partial agonist at the ER. However, the mechanism of how MCDF interacts with the ER is not clear. Competition binding assays were performed to determine whether MCDF could...
This indicated that both E2- and MCDF-induced ER shifted bands appeared that were also supershifted by the ER antibody G20. When MCDF and the ER were combined, similar shifted bands were observed. Similar results were observed in MCF-7 cells cotransfected with pERE-luciferase and a nonspecific scrambled RNA (iSc). In contrast, cotransfection with a siRNA that targets the luciferase expression plasmid (to enhance inducibility), and iSc, iGL2, or iAhR. Results from the RNA inter-

interact directly with the ER by competing with [3H]E2 for ER binding. Diethylstilbestrol competed away the binding of 0.1 nM [3H]E2 to the ER, whereas MCDF was not able to compete with [3H]E2 (data not shown). However, MCDF induced an interaction of GST-HBD (a fusion protein of GST and hormone-binding domain of ER; Ref. 30) with a nuclear receptor coactivator, AIB1 (31), in GST pull-down assays as shown in Fig. 6. ICI 182,780 blocked both E2- and MCDF-induced ER-AIB1 interaction. This result suggests that MCDF directly binds to ER and induces the formation of ER-coactivator complex and activates expression of ER-regulated genes.

Examination of E2- and MCDF-Induced ER-ERE Complex Formation Using Gel Mobility Shift Assays. To further address the mechanism by which MCDF interacts with the ER, gel mobility shift experiments were performed. In vitro-transcribed/translated ER, AhR, and Arnt proteins were used in various combinations to determine whether they formed a complex on an ERE (Fig. 7A). The rabbit reticulolysate contains a variety of proteins in addition to the in vitro-translated protein. To test whether the mobility shifts observed were due to the direct binding of the MCDF-ER complex to an ERE, a gel mobility shift was performed using purified ER (Fig. 7C). A concentration-dependent increase in the shifted complex was observed when the pure ER was combined with 1 nM and 10 nM E2. The formation of the 10 nM E2-ER complex was inhibited by ICI 182,780, and the complex was supershifted using the G20 antibody. Similarly, a concentration-dependent increase in the amount of the MCDF-ER complex was observed. This complex was also supershifted using the G20 antibody, and complex formation was prevented in the presence of ICI 182,780. These gel mobility shift data suggested that MCDF bound directly to the ER, resulting in the formation of a complex with an ERE.

A recent study (22) proposed that AhR ligands such as 3MC exhibit estrogenic activity through AhR-ER interactions with ERs. The potential role of these interactions in mediating the estrogenic activity of MCDF was further investigated in MCF-7 cells transfected with siRNA for the AhR, which causes a >80% knockdown in the transfected cells (32). The results illustrated in Fig. 8A show that both E2 and MCDF induce luciferase activity in MCF-7 cells cotransfected with pERE-luciferase and a nonspecific scrambled RNA (iSc). In contrast, cotransfection with a siRNA that targets the luciferase mRNA (iGL2) resulted in the loss of inducibility by E2 and MCDF. Knockdown of the AhR with iAhR did not affect inducibility by E2 or MCDF, and slightly higher luciferase activities induced by MCDF were observed. Similar results were observed in MCF-7 cells cotransfected with pERE-luciferase, ERα expression plasmid (to enhance inducibility), and iSc, iGL2, or iAhR. Results from the RNA inter-
ference studies complement the previous study showing the estrogenic activity of TCDD (32) and support a mechanism in which MCDF directly activates ER.

**DISCUSSION**

In all of the assays used, MCDF was shown to induce typical E2-like responses at concentrations of 1–10 μM in the presence of the human wt-ER. Western blots indicated that E2, MCDF, and ICI 182,780 treatment degraded ER protein in MCF-7 cells (Fig. 2). ER degradation at the protein level by MCDF has been described previously (12, 16, 17), and our results are consistent with these reports. Analysis of ER mRNA levels indicated that E2 decreased ER mRNA, whereas MCDF and ICI 182,780 had no effect (data not shown). Therefore, although all three compounds result in ER protein down-regulation, only E2 treatment results in ER degradation at the mRNA level. Despite the differences in ER mRNA regulation, the degradation of ER protein by E2, MCDF, and ICI 182,780 occurs posttranslationally and has been shown to be mediated by the proteasome (16, 33–36). It is possible that MCDF induces proteasome-dependent degradation of the ER through both the ER and the AhR (16, 37).

Growth assays in MCF-7 cells showed that a 1 μM concentration of MCDF stimulated cell growth, and E2 was also able to stimulate cell growth (Fig. 3). MCDF also acted as an antiestrogen at a 1 μM concentration and was able to partially block the E2 response. ICI 182,780 degrades the ER and was therefore able to completely block the E2 response. ICI 182,780 treatment degraded ER protein in MCF-7 cells (Fig. 2). ER degradation indicated that the ER was required for MCDF-mediated induction of cell growth. Luciferase assays using a transiently transfected ERE-luciferase reporter were performed in T47D:C4:2 cells and MCF-7 cells (Fig. 4). Higher concentrations of MCDF (0.1–1 μM) resulted in an induction of luciferase activity, and MCDF was antiestrogenic in that it was able to partially block the E2-induced response. Two lines of evidence indicated that the ER was required for MCDF-mediated induction of an ERE-luciferase reporter. The first was that when the ER was degraded on ICI 182,780 treatment, no MCDF induction was observed. The second was that no MCDF response was observed in ER-negative T47D:C4:2 cells transfected with the reporter alone. However, MCDF induction was restored when the ER was cotransfected with the reporter. A similar effect was observed in ER-negative MDA-MB-231 human breast cancer cells. MDA-MB-231 cells are AhR nonresponsive, but when the ER was transfected into these cells, AhR responsiveness was restored (38).

Northern blots indicated that MCDF was able to induce the E2-responsive gene, TGF-α (Fig. 5). The ER is required for the MCDF-mediated induction because ICI 182,780 completely inhibited transcription of TGF-α. TCDD has also been shown to induce TGF-α mRNA in the human keratinocyte cell line SCC-12F (39).

Overall, these data support the idea that MCDF is a partial agonist at the ER that has antiestrogen action at higher concentrations. Another AhR agonist that exhibits both estrogenic and antiestrogenic activity is indolo[3,2-b]carbazole (40). Indolo[3,2-b]carbazole is an acid-derived condensation product of indole-3-carbinol, which is found in various vegetables, that binds to the AhR and the ER. Furthermore, TCDD, a compound structurally related to MCDF, has estrogenic activity in MCF-7 cells transfected with siRNA for the AhR (32).
Many studies have described potential AhR-ER cross-talk, but a precise mechanism of action has not been elucidated. Nevertheless, potential mechanisms accounting for the antiestrogenic action of MCDF have been suggested (41). First of all, AhR-mediated induction of cytochrome P4501A1 results in an increased metabolism of E2 (2). Second, down-regulation of the ER by AhR agonists could contribute to antiestrogen action (42). Finally, possible interactions could occur at the level of transcription. AhR agonists could interfere with ER transcriptional activation by inhibiting ligand and/or DNA binding and competing for common coactivators. For example, the ER and AhR interact with the coactivator receptor-interacting protein 140, suggesting that the ER and AhR share at least one common coactivator (43). Certain E2-responsive genes promoters, such as those of c-fos, cathepsin D, pS2, and progesterone receptor, contain overlapping DRE and ERE DNA sequences, suggesting that competition for DNA sites could occur (44).

The estrogenic properties of MCDF can be explained by the results of the GST pull-down (Fig. 6), the ERE mobility shift assay (Fig. 7), and RNA interference studies (Fig. 8). The GST pull-down assays and gel mobility shift data indicated that MCDF bound directly to the ER, induced ER-AIB1 interaction, and produced a complex that binds to an ERE. The formation of the MCDF-ER complex is blocked by ICI 182,780, and the presence of the ER is confirmed because it is supershifted by an ER antibody. It is interesting to note that the E2/ER-ERE complex migrated faster than the MCDF-ER-ERE complex, suggesting that each ligand may induce a different ER conformation. In contrast, both E2 and MCDF activated pERE-luciferase in the presence or absence of cotransfected iAhR (Fig. 8).

The ability of MCDF to act directly through the ER is supported by computer modeling predictions. Using modeling programs, the molecular structures of E2 and MCDF were overlayed (Fig. 9A). E2 and MCDF were found to occupy equivalent volumes of 237 and 249.8 cubic angstroms, respectively. E2 (Fig. 9B) and MCDF (Fig. 9C) were docked into the ER ligand-binding domain, and both ligands fit into the ER-binding pocket. This is not surprising because the volume of the binding cavity is nearly twice that of its cognate ligand (45).

Using competition binding assays, MCDF did not compete with E2 for binding to the ER (data not shown). Previous studies in rats (14) have also shown that MCDF did not compete with radiolabeled E2 for uterine ER binding sites. One possible explanation is that MCDF could bind to the ER at a site other than the classical ER binding site. Alternate ligand-binding sites have been identified in ER for the ligand tetrahydrochrysene (46) and estrogen receptor (47). If MCDF binds to an alternate binding site other than E2, this could explain the inability of MCDF to block E2 or diethylstilbestrol action in competition binding assays. A second possibility is that MCDF has a weak affinity for the ER. This interaction is not detected by competition binding assays, but the activity of MCDF can be detected in assays such as growth assays, luciferase assays, GST pull-down assays, and gel mobility shifts. There are no hydroxyls to tether MCDF appropriately to aid in receptor folding. Nevertheless, if a low-affinity ligand does bind because of its hydrophobic nature, coactivator molecules such as AIB1 could stabilize the complex to initiate a transient growth response (48). This principle can also be extended to certain phytoestrogens that exhibit weak ER binding (49, 50) but possess estrogenic activity (51).

A recent report by Ohtake et al. (22) showed that the AhR ligand 3MC activated an ERE-luciferase reporter. The activation required the formation of a complex between the ER, AhR, Arnt, and 3MC. In the proposed model, 3MC binds to the AhR, and the complex translocates to the nucleus, where subsequent recruitment of Arnt and the unliganded ER to the AhR occurs. In our model, MCDF interacts directly with the ER, and the AhR and Arnt are not required, as shown in gel shift assays (Fig. 7) and RNA interference (Fig. 8) assays in MCF-7


Interaction of the Aryl Hydrocarbon Receptor Ligand
6-Methyl-1,3,8-trichlorodibenzofuran with Estrogen Receptor
$\alpha$

Sandra Timm Pearce, Hong Liu, Ishwar Radhakrishnan, et al.


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