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IN VITRO INHIBITION OF 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN-INDUCED ACTIVITY BY α-NAPHTHOFLAVONE AND 6-METHYL-1,3,8-TRICHLORODIBENZOFURAN USING AN ARYL HYDROCARBON (Ah)-RESPONSIVE CONSTRUCT

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Abstract—Rat hepatoma H4IIE and mouse hepatoma Hepa 1c1c7 cells were transiently transfected with a plasmid construct that contained the bacterial chloramphenicol acetyltransferase (CAT) gene under the control of the mouse mammary tumor virus promoter and one copy of the dioxin responsive element. Treatment of transfected H4IIE and Hepa 1c1c7 cells with 10^{-13} to 10^{-6} M 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) resulted in a concentration-dependent increase in transient CAT activity. Maximum CAT activity was induced in both cell lines by exposure to 10^{-9} M TCDD. The induction of CAT activity correlated well with the TCDD-induced, P4501A1-dependent ethoxyresorufin *O*-deethylase activity. Cotreatment of transfected cells with 10^{-9} M TCDD and 10^{-8} to 10^{-6} M α-naphthoflavone (αNF) or 6-methyl-1,3,8-trichlorodibenzofuran (MCDF) resulted in a concentration-dependent reduction of TCDD-induced CAT activity. Treatment of cells with 10^{-6} M αNF or MCDF alone resulted in only minimal induction of CAT activity. Both antagonists inhibited the induction of genes under the control of the CYP1A1 and mouse mammary tumor virus promoters, which indicates that the αNF- and MCDF-mediated antagonism of TCDD-induced, aryl hydrocarbon receptor-dependent gene transcription does not depend on promoter context.

Key words: α-naphthoflavone; TCDD; inhibition; CYP1A1; Ah-responsive constructs; 6-methyl-1,3,8-trichlorodibenzofuran

TCDD† has been used extensively as a prototype to investigate the mechanism of the toxic and biological effects elicited by the toxic halogenated aromatic hydrocarbons [1–3]. TCDD elicits a wide variety of toxic and biochemical responses both *in vitro* and *in vivo*. The induction of *CYP1A1* gene expression by TCDD involves the binding of TCDD to the AhR. The liganded AhR complex then forms a heterodimer with the aryl hydrocarbon nuclear translocator (Arnt) protein to form a nuclear complex, which interacts with DREs located in the 5′ regulatory region of the *CYP1A1* gene [3].

A variety of compounds that bind to the AhR are weak AhR agonists for many of the induced responses. For example, Luster and coworkers [4] demonstrated that 1-amino-3,7,8-trichlorodibenzo-*p*-dioxin antagonizes TCDD-induced myelotoxicity and displaces [^{3}H]TCDD from the AhR with an EC_{50} of 3.7 nM. In addition, the amino compound antagonized TCDD-mediated induction of EROD activity. Other studies have shown that several polychlorinated biphenyl congeners partially antagonize TCDD-mediated immunotoxicity and teratogenicity [5–7].

Some synthetic flavones inhibit the carcinogenic effects of polynuclear aromatic hydrocarbons such as 7,12-dimethylbenzanthracene and benzo[*a*]pyrene [8, 9]. The synthetic flavonoid αNF (7,8-benzoflavone) is an inhibitor of some TCDD-induced microsomal P450 enzyme activities [10, 11]. It has also been reported that αNF is metabolized by microsomal P450 enzymes, which may explain its low toxicity *in vivo* [12–14] and weak clastogenic activity [15, 16]. αNF antagonizes TCDD- and benzo[*a*]pyrene-mediated immunosuppression and binds to the AhR with moderate affinity [17, 18]. In addition, results from our laboratory have indicated that αNF competes with TCDD for binding to the cytosolic AhR in rat, mouse, and human hepatoma cell lines [19]. Furthermore, treatment of cells with TCDD and αNF ($\leq 1 \times 10^{-6}$ M) results in inhibition of TCDD-induced *CYP1A1* gene expression, and this is paralleled by decreased formation of the nuclear AhR complex as determined using a radioligand binding or gel retardation assay [20, 21]. However, treatment of rat and murine hepatoma cell lines with 10^5 M αNF results in induction of AhR- and DRE-dependent *CYP1A1* gene

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† Abbreviations: AHH, aryl hydrocarbon hydroxylase; AhR, aryl hydrocarbon receptor; αNF, α-napthoflavone; CAT, chloramphenicol acetyltransferase; DRE, dioxin responsive element; EROD, ethoxyresorufin *O*-deethylase; MCDF, 6-methyl-1,3,8-trichlorodibenzofuran; α-MEM, α-Minimum Essential Medium; and TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

transcription, demonstrating that αNF is an AhR agonist at concentrations $>10^{-6}$ M [22].

MCDF has been identified as an antagonist of TCDD-induced EROD and AHH activities in rats, mice and transformed mammalian cells in culture [23–26]. MCDF is a weak inducer of these activities at high concentrations (10^{-6} M) despite its relatively high affinity for the AhR ($K_D \approx 10$ nM) [27]. MCDF antagonizes TCDD-mediated induction of *CYP1A1* gene transcription by inhibiting formation of the [^{3}H]TCDD–nuclear AhR complex. However, incubation of nuclear extracts from cells treated with 10^{-6} M MCDF and ^{32}P-labeled DRE oligonucleotide results in formation of a DRE–protein complex, which is observed as a retarded band in a gel electrophoretic mobility shift assay [20, 21]. This study investigates the comparative partial AhR antagonist activity of αNF and MCDF in transient transfection assays using expression constructs that contain a mouse DRE, a mouse mammary tumor virus promoter, and a bacterial CAT reporter gene.

MATERIALS AND METHODS

Chemicals and biochemicals. TCDD, [^{3}H]TCDD (37 mCi/μmol), ethoxyresorufin, and MCDF were synthesized in this laboratory to >98% purity as determined by gas chromatographic analysis. D-Threo-[*dichloroacetyl*-1-^{14}C]chloramphenicol (54 mCi/mmol) was purchased from the Amersham Corp. (Arlington Heights, IL). [α-^{32}P]Deoxycytosine triphosphate (3000 mCi/μmol) was purchased from New England Nuclear Research Products (Boston, MA). All other chemicals and biochemicals were of the highest purity available from commercial sources.

Treatment and maintenance of cells. Rat hepatoma H4IIE and mouse hepatoma Hepa 1c1c7 cells were grown as continuous cell lines in α-MEM supplemented with 2.2 mg/mL tissue culture grade sodium bicarbonate, 5% fetal bovine serum (v/v), and 10 mL/L antibiotic/antimycotic solution (Sigma, St. Louis, MO). Stock cultures of all cell lines were grown in 150-cm^2 tissue culture flasks and incubated in a humidified mixture of 5% carbon dioxide and 95% air under atmospheric pressure. After reaching confluency, the cells were trypsinized and diluted to approximately 10^6 cells/mL. For enzyme assays, approximately 2×10^6 cells in 5 mL of medium were passaged to 25-cm^2 tissue culture flasks. Solutions of the inducers and antagonists dissolved in DMSO were added to the flasks so that the final concentration of DMSO in the medium was 0.5 to 1%. Cells were harvested and assayed for CAT and EROD activity 24 hr after dosing.

Cytochrome P4501A1 enzyme assays. Cells were isolated by manual scraping from the plate, and the EROD activities were determined by the method of Pohl and Fouts [28] as previously described. Cells were harvested in Tris-sucrose buffer (20 mM sucrose, 0.25 M Tris–HCl, pH 8.0) and centrifuged at 1000 *g* for 5 min at 2°. The cell pellet was resuspended in 150 μL Tris-sucrose buffer. The cell suspension was aliquoted (50 μL) into fresh 16 × 100 mm glass tubes in duplicate, and the remainder was frozen until ready for protein determination. To the cell suspension was added 1.15 mL of cofactor solution (0.7 mg NADPH, 0.7 mg NADH, 0.7 mg BSA, 1.4 mg $MgSO_4$). The mixtures were warmed in a 37° water bath for 2 min, and the reactions were initiated at 10-sec intervals by the addition of 50 μL of ethoxyresorufin substrate. The reactions were allowed to continue for 15 min and were terminated by the addition of 2.5 mL of MeOH. The samples were centrifuged for 5 min to pellet the flocculated protein, and the clear supernatants were quantitated for resorufin spectrofluorimetrically (excitation $\lambda = 550$ nm, emission $\lambda = 585$ nm, slit width = 6.5 nm). The fluorescence of each sample was compared to a standard curve to determine the concentration of resorufin. Protein concentrations were determined by the method of Bradford [29], using BSA as a standard.

Plasmid preparation. The plasmid pMCAT 5.12 is a construct provided by Dr. J. P. Whitlock, Jr. (Stanford University), which contains the mouse dioxin responsive element 2 (DRE 2) fused to the mouse mammary tumor virus promoter driving the CAT reported gene [30, 31]. The plasmid in *Escherichia coli* HB101 was grown in nutrient broth supplemented with 50 mg/L ampicillin, extracted by alkaline lysis, and purified via two rounds of cesium chloride equilibrium centrifugation by the methods of Sambrook *et al.* [32]. The purified plasmid DNA was removed from the gradients, and the intercalated ethidium bromide was extracted seven times with *n*-butanol. The resulting plasmid solution was quantitated by absorbance at 260 nm, and 1 μg was electrophoresed on a 1% agarose gel to verify the purity of the preparation.

DNA transfection. Rat hepatoma H4IIE and mouse hepatoma Hepa 1c1c7 cells were grown to approximately 80% confluency in 150-cm^2 tissue culture flasks as previously described [20]. pMCAT 5.12 (120 μg) was added dropwise to 500 μL of 2 HEPES-buffered saline (40 mM HEPES, 0.28 M sodium chloride, 1.5 mM Na_2HPO_4, pH 7.05) containing 62 μL of 2 M calcium chloride. The solution was diluted to 1 mL with distilled water, and the plasmid DNA was allowed to precipitate undisturbed for 30 min. Immediately prior to transfection, the α-MEM was removed from the tissue culture plates and replaced with 15 mL α-MEM without fetal bovine serum. Approximately 100 μL of DNA preparation containing 120 μg of pMCAT 5.12 was added to each 150-cm^2 tissue culture flask. After 4 hr, the DNA suspension was removed, and the cells were exposed to 20% DMSO : 80% α-MEM (v/v) solution for 4 min, rinsed thoroughly twice with α-MEM, and allowed to grow for 12 hr. The cells were then harvested by trypsinization and plated into 25-cm^2 tissue culture flasks (approximately 10^6 cells/flask). After 8–12 hr, cells were treated with chemicals.

CAT assay. After treatment with the chemicals for 24 hr, cells were harvested by manual scraping from the plate, centrifuged at 1000 *g* for 5 min at 2°, and resuspended in 20 μL of 0.25 M Tris–HCl (pH 7.8). Cells were disrupted by exposure to three freeze–thaw cycles (1 min acetone–dry ice and 1 min 37° water bath, respectively), the homogenate was vortexed for 15 sec, and the cell debris was centrifuged at 10,000 *g* for 5 min at 2°. The clear

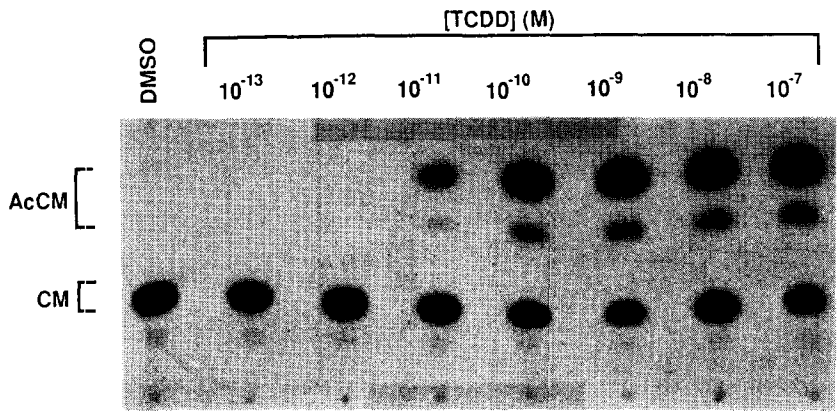

Fig. 1. Concentration-dependent induction of CAT activity by TCDD in H4IIE cells. Cells were transfected with pMCAT 5.12, treated with chemicals for 24 hr, harvested, and assayed for CAT activity as described in Materials and Methods. The unreacted chloramphenicol (CM) and acetylated metabolites (AcCM) are indicated in the chromatogram.

supernatant was extracted with a Hamilton syringe and placed into a fresh tube, and the volume was adjusted to 105 μL with 0.25 M Tris–HCl (pH 7.8). Samples were heated to 65° for 10 min, and then allowed to cool. Seventy-five microliters of sample was placed into a fresh tube, and the rest was frozen for protein determination. The reaction was initiated by the addition of 20 μL of a freshly prepared solution of [^{14}C]chloramphenicol (54 mCi/mmol, 20 μM) and acetyl CoA (4 mM). The reaction was allowed to continue at 37° with gentle mixing of samples at 10-min intervals, and then was terminated by the addition of 100 μL of ethyl acetate followed by vortexing for 10 sec. Samples were centrifuged at 10,000 *g* for 5 min, and the upper organic phase was removed. The aqueous phase was reextracted with 100 μL of ethyl acetate, and the extracts from each sample were pooled and evaporated in a 70° water bath under a hood. The residue was dissolved in 20 μL of ethyl acetate and spotted on Whatman TLC plates. The samples were placed in TLC chambers containing chloroform:methanol (95:5) and retrieved when the solvent front was approximately 2–3 cm from the top of the plate. CAT activity was determined by quantitating acetylated and unacetylated [^{14}C]chloramphenicol on a Betagen Betascope 603 blot analyzer imaging system and visualized by autoradiography. Quantitation of the CAT assay activities was carried out using incubation times and protein concentrations in which <20% acetylation was observed, and this was in the linear range for this activity.

Statistical analysis. EROD activities are reported as picomoles product formed per minute per milligram of protein. CAT activities are reported as percent maximum activity for each experiment. Results for all studies are presented as the means ± SD for at least three determinations for each treatment group. Multiple comparisons for all experiments were made using ANOVA and Sheffe's post hoc comparisons (MacIntosh Super ANOVA™ software package).

RESULTS

In rat hepatoma H4IIE cells transfected with pMCAT 5.12, TCDD induced CAT activity at concentrations as low as 10^{-11} M with maximum activity induced at a concentration of 10^{-9} M (Fig. 1). The EC_{50} value for induction of CAT activity by TCDD was 3×10^{-11} M. Figure 2 summarizes the concentration-dependent effects of TCDD, αNF and MCDF on the induction of CAT activity in H4IIE cells. In contrast to TCDD, MCDF and αNF induced only minimal activity (10 and 17%, respectively) of maximal induction at the highest concentrations tested (10^{-6} M). The results in Table 1 summarize the effects of αNF and MCDF on TCDD-mediated

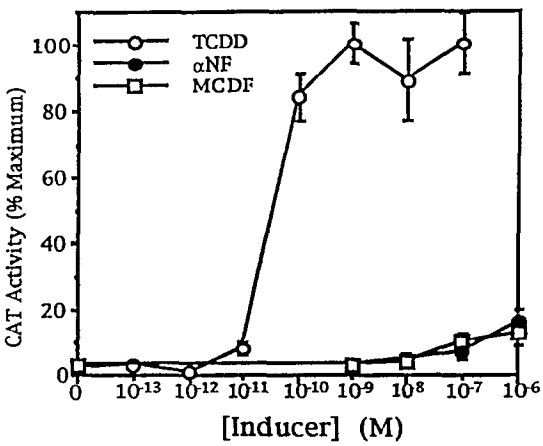

Fig. 2. Effects of TCDD, αNF and MCDF on the induction of CAT activity in H4IIE cells. Cells were transfected with pMCAT 5.12, treated with chemicals for 24 hr, harvested, and assayed for CAT activity as described in Materials and Methods. The results are expressed as means ± SD for at least three separate determinations for each data point.

Table 1. Effects of TCDD and TCDD plus αNF or MCDF on CAT activity in rat hepatoma H4IIE cells transiently transfected with pMCAT 5.12*

Treatment	CAT activity (% maximum)
TCDD (10^{-9} M)	100
TCDD (10^{-9} M) + αNF (10^{-8} M)	78 ± 5†
TCDD (10^{-9} M) + αNF (10^{-7} M)	53 ± 6†
TCDD (10^{-9} M) + αNF (10^{-6} M)	24 ± 4†
αNF (10^{-6} M)	16 ± 4†
DMSO	4 ± 1†
TCDD (10^{-9} M)	100
TCDD (10^{-9} M) + MCDF (10^{-8} M)	42 ± 7†
TCDD (10^{-9} M) + MCDF (10^{-7} M)	21 ± 5†
TCDD (10^{-9} M) + MCDF (10^{-6} M)	10 ± 3†
MCDF (10^{-6} M)	7 ± 4†
DMSO	5 ± 2†

* H4IIE cells were transfected with pMCAT 5.12, treated with chemicals for 24 hr, harvested, and assayed for CAT activity as described in Materials and Methods. The results are expressed as means ± SD for at least three separate determinations for each data point. MCDF and TCDD do not directly inhibit CAT activity with diverse AhR-independent constructs using diverse promoters (data not shown).

† Significantly lower than TCDD-induced CAT activity as determined by ANOVA ($P < 0.05$).

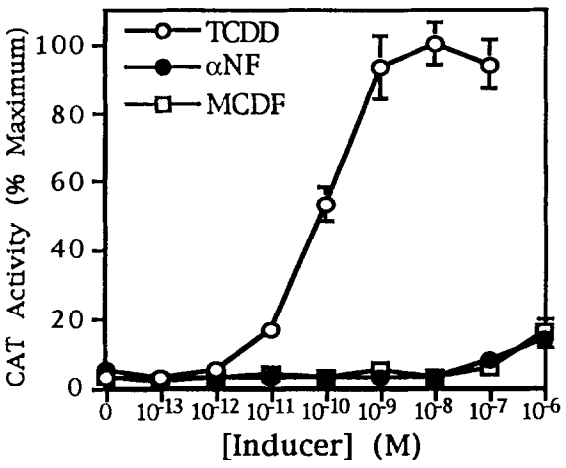

Fig. 3. Effects of TCDD, αNF and MCDF on the induction of CAT activity in Hepa 1c1c7 cells. Cells were transfected with pMCAT 5.12, treated with chemicals for 24 hr, harvested, and assayed for CAT activity as described in Materials and Methods. The results are expressed as means ± SD for at least three separate determinations for each data point.

induction of CAT activity in H4IIE cells. Cotreatment of cells with TCDD (10^{-9} M) and αNF (10^{-8} to 10^{-6} M) resulted in a concentration-dependent decrease in TCDD-induced CAT activity. In contrast, the CAT activity in cells treated with 10^{-6} M αNF alone was only 16% of the maximum value. Treatment of cells with TCDD (10^{-9} M) and MCDF (10^{-8} to 10^{-6} M) resulted in a 58, 79 and 90% inhibition of TCDD-induced CAT activity, respectively. However, treatment of cells with 10^{-6} M MCDF alone resulted in only 7% of maximal induction of CAT activity.

The results in Fig. 3 illustrate the concentration-dependent induction of CAT activity by TCDD, αNF and MCDF in Hepa 1c1c7 cells. TCDD induced CAT activity at concentrations as low as 10^{-11} M, and maximum activity was induced at 10^{-9} M. The EC_{50} value for induction of CAT activity by TCDD was approximately 8×10^{-11} M. MCDF and αNF induced only minimal activity (10 and 8%, respectively) of the maximally induced value at the highest concentrations tested (10^{-6} M). Table 2 summarizes the effects of αNF and MCDF on the induction of CAT activity by TCDD in Hepa 1c1c7 cells. Treatment of cells with TCDD (10^{-9} M) and αNF (10^{-8} to 10^{-6} M) resulted in a 22, 59 and 87% decrease, respectively, in TCDD-induced CAT activity. Treatment of cells with TCDD (10^{-9} M) and MCDF (10^{-8} to 10^{-6} M) produced a 38, 68 and 86% inhibition of CAT induction, respectively. In contrast, CAT activity in cells treated with 10^{-6} M MCDF alone was only 12% of the activity induced by 10^{-9} M TCDD in Hepa 1c1c7 cells.

Table 2. Effects of TCDD and TCDD plus αNF or MCDF on CAT activity in mouse Hepa 1c1c7 cells transiently transfected with pMCAT 5.12*

Treatment	CAT activity (% maximum)
TCDD (10^{-9} M)	100
TCDD (10^{-9} M) + αNF (10^{-8} M)	78 ± 6†
TCDD (10^{-9} M) + αNF (10^{-7} M)	41 ± 7†
TCDD (10^{-9} M) + αNF (10^{-6} M)	13 ± 2†
αNF (10^{-6} M)	9 ± 1†
DMSO	11 ± 1†
TCDD (10^{-9} M)	100
TCDD (10^{-9} M) + MCDF (10^{-8} M)	62 ± 9†
TCDD (10^{-9} M) + MCDF (10^{-7} M)	32 ± 11†
TCDD (10^{-9} M) + MCDF (10^{-6} M)	14 ± 4†
MCDF (10^{-6} M)	12 ± 2†
DMSO	3 ± 0†

* The experimental procedures were comparable to those used for the H4IIE cells (Table 1).

† Significantly lower than TCDD-induced CAT activity as determined by ANOVA ($P < 0.05$).

DISCUSSION

Reporter gene constructs have been used extensively to study the regulation of eukaryotic gene expression [33–35]. Deletion analysis of the 5′-promoter region of the mouse *CYP1A1* gene has been used to characterize the AhR-dependent induction of *CYP1A1* gene expression and to identify *cis*-acting genomic DRE sequences [30, 31, 36]. The plasmid construct pMCAT 5.12 contains the *CAT* gene linked to the mouse mammary tumor virus (MMTV) promoter without the associated glucocorticoid responsive element. A single copy of DRE 2, isolated from mouse genomic DNA upstream

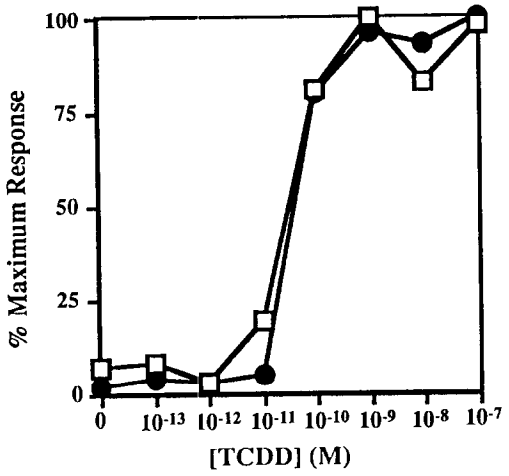

Fig. 4. Correlation between TCDD-induced EROD (●) and CAT (□) activities in H4IIE cells.

(−1177 to −2263) from the *CYP1A1* gene, has been cloned into the plasmid construct [30]. Transfection of H4IIE and Hepa 1c1c7 cells with the plasmid pMCAT 5.12 resulted in a eukaryotic system that transiently expressed bacterial CAT activity in response to TCDD and related compounds. Treatment of transfected H4IIE cells with different concentrations of TCDD resulted in a concentration-dependent increase in CAT activity in both cell lines (Figs. 2 and 3). Maximum CAT activity was induced in both cell lines at a concentration of 10^{-9} M TCDD, and the EC_{50} values for induction in H4IIE and Hepa 1c1c7 were 30 and 80 pM, respectively. Induction of CAT activity correlated well with induction of EROD activity in H4IIE cells [20] (Fig. 4). CAT activity was induced approximately 3.5-fold higher in H4IIE cells (relative to control values) than in Hepa 1c1c7 cells, and these results correlated with the higher levels of EROD activity induced in H4IIE cells [20]. However, the higher levels of induced CAT activity in H4IIE cells could be a result of higher transfection efficiency for this cell line. Cotreatment of transiently transfected H4IIE and Hepa 1c1c7 cells with 10^{-9} M TCDD and αNF or MCDF (10^{-8} to 10^{-6} M) resulted in a concentration-dependent decrease in TCDD-induced CAT activity by both antagonists, and similar results were observed for inhibition of EROD activity and P4501A1 mRNA levels in these cells [20]. The inhibitory responses correlated with decreased nuclear AhR levels in cells cotreated with TCDD plus MCDF or αNF [20, 21]. Since both antagonists inhibited induction of transcription of genes under the control of the CYP1A1 and MMTV promoters, these data indicate that the αNF- and MCDF-mediated antagonism of TCDD-induced, AhR-dependent gene expression is not promoter dependent.

In summary, the results of this study confirm that both αNF and MCDF inhibit induction of transient CAT activity in Hepa 1c1c7 and H4IIE cells transfected with the DRE-containing pMCAT 5.12 plasmid. Previous studies have reported that formation of a radiolabeled nuclear AhR complex is inhibited in cells cotreated with [^{3}H]TCDD and 10^{-6} M MCDF or αNF [20, 21], and this is consistent with the AhR antagonist activity of both compounds. Recent studies indicate that the AhR antagonist activity of αNF involves AhR sequences that are also important for TCDD transactivation responses [37]. The regions of the AhR required for the antagonist effects of MCDF have not been defined. It would appear that MCDF is both a partial AhR agonist and antagonist since a 10^{-6} M concentration of this compound alone is a potent inducer of AhR-mediated antiestrogenic activity in both *in vivo* and *in vitro* models [27, 38]. The molecular biology of the partial agonist/antagonist activity of MCDF is currently being investigated.

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