0026-895X/93/020200-07$03.00/0

MOLECULAR PHARMACOLOGY, 43:200–206



# α-Naphthoflavone-Induced *CYP1A1* Gene Expression and Cytosolic Aryl Hydrocarbon Receptor Transformation

M. SANTOSTEFANO, M. MERCHANT, L. ARELLANO, V. MORRISON, M. S. DENISON, and S. SAFE

*Department of Veterinary Physiology and Pharmacology, Texas A & M University, College Station, Texas 77843–4466 (M.S., M.M., L.A., V.M., S.S.), and Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824 (M.S.D.)*

Received June 30, 1992; Accepted November 6, 1992

**SUMMARY**

α-Naphthoflavone (αNF) is a weak aryl hydrocarbon (Ah) receptor agonist and inhibits the induction of *CYP1A1* gene expression by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. It has been suggested that the Ah receptor antagonist activity is due to the formation of αNF-cytosolic Ah receptor complexes that fail to undergo transformation. This hypothesis is consistent with data obtained in this and other studies using αNF concentrations from 10 to 1000 nM. However, 10 μM αNF exhibited Ah receptor agonist activity in several assays. Incubation of rat hepatic cytosol with 10 μM αNF caused transformation of the Ah receptor, as determined in a gel retardation assay using a $^{32}$P-labeled oligonucleotide containing a single dioxin-responsive element (DRE). Incubation of rat hepatoma (H-4-II E) cells with 10 μM αNF not only resulted in the induction of *CYP1A1* mRNA levels but also increased chloramphenicol acetyltransferase activity from a DRE-containing chloramphenicol acetyltransferase reporter plasmid. Moreover, the DRE-transformed cytosolic Ah receptor complex liganded with either αNF or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin did not undergo significant dissociation at 4°. These data confirm that αNF is an Ah receptor agonist and, based on the results of previous studies, exhibits partial antagonist activity via competition for receptor binding sites.

TCDD elicits a diverse spectrum of tissue-, age-, sex-, species-, and cell-specific biochemical and toxic responses in laboratory animals and mammalian cells in culture (1–4). It has been proposed that many TCDD-induced effects are mediated through initial binding to an intracellular cytosolic protein, namely the Ah receptor, and there is strong evidence, in the form of genetic, structure-activity, and molecular biology studies, that supports the proposed receptor-mediated signal transduction mechanism (1–4). Extensive molecular biology studies on the induction of *CYP1A1* gene expression by TCDD and related aromatic hydrocarbons have shown that the liganded cytosolic Ah receptor complex undergoes transformation and nuclear translocation. The resulting heterodimeric nuclear Ah receptor complex binds to *cis*-acting genomic sequences (DREs) upstream from the *CYP1A1* gene and this interaction results in enhanced gene transcription (5–8). The overall induction process is similar to that described for the steroid hormones; however, the results of recent studies (9–11) on the structure of the two proteins that comprise the nuclear heterodimeric complex, namely the Ah receptor and nuclear translocator proteins, indicate that this nuclear transcription factor is not a member of the steroid and thyroid hormone receptor superfamily (12).

Research in several laboratories has demonstrated that different structural classes of compounds, including substituted dibenzo-*p*-dioxins and dibenzofurans, polychlorinated biphenyls, and αNF, exhibit Ah receptor antagonist activity (13–26). For example, MCDF and αNF have been extensively investigated in this laboratory as inhibitors of TCDD-mediated induction of *CYP1A1* gene expression (18–26). In receptor binding assays, both compounds competitively displaced [$^3$H]TCDD from the cytosolic Ah receptor and decreased formation of the nuclear [$^3$H]TCDD-Ah receptor complex. In contrast, 1 μM concentrations of αNF, but not MCDF, decreased the DRE binding of nuclear extracts from mammalian transformed cell lines cotreated with 1 nM TCDD plus the antagonists (23, 25, 26). The results obtained for MCDF are consistent with the partial agonist activity of this compound as an antiestrogen (27). Gasiewicz and Rucci (28) recently reported that αNF inhibits the TCDD-induced transformation of the rat hepatic cytosolic receptor, and they suggested that the cytosolic Ah receptor complex liganded with αNF does not undergo transformation due to the altered conformation of the protein-αNF

This work was supported by the Texas Agricultural Experiment Station and the National Institutes of Health (Grant ES03843). S.S. is a Burroughs Wellcome Toxicology Scholar.

**ABBREVIATIONS:** TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; NF, naphthoflavone; DRE, dioxin-responsive element; MCDF, 6-methyl-1,3,8-trichlorodibenzofuran; TCDF, tetrachlorodibenzofuran; $^{125}$I-MCDF, 8-[$^{125}$I]iodo-6-methyl-1,3-dichlorodibenzofuran; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; α-MEM, α-minimum essential medium; DMSO, dimethylsulfoxide; kb, kilobase(s); P450, cytochrome P450; CAT, chloramphenicol acetyltransferase.

complex. αNF also antagonizes benz[a]anthracene-induced aryl hydrocarbon hydroxylase activity in HeLa cells and hamster embryo cells in culture (29, 30) and this may be due, in part, to inhibition of enzyme activity. This study further investigates the structural and functional properties of αNF as an Ah receptor ligand and demonstrates that this compound is a partial agonist that induces both the *in vitro* transformation of the rat cytosolic Ah receptor complex and *CYP1A1* gene expression in cultured rat hepatoma H-4-II E cells.

## Materials and Methods

**Chemicals and biochemicals.** TCDD was synthesized in this laboratory to >98% purity, as determined by gas chromatographic analysis, and αNF was purchased from Sigma Chemical Co. (St. Louis, MO). The cell line was obtained from the American Type Culture Collection. All other chemicals and biochemicals were of the highest purity available from commercial sources. The following buffers were used: HED (25 mM HEPES, 1.5 mM EDTA, 1 mM dithiothreitol, pH 7.6), HEDG (HED supplemented with 10%, v/v, glycerol, pH 7.6), and HDK (1 mM dithiothreitol, 0.4 M KCl, 25 mM HEPES, pH 2.5).

**Treatment and maintenance of cells.** Rat hepatoma H-4-II E cells were grown as a continuous cell line in α-MEM supplemented with 2.2 mg/ml tissue culture-grade sodium bicarbonate, 5% (v/v) fetal calf serum, and 10 ml/liter antibiotic/antimycotic solution. Stock cultures were grown in 150-cm$^2$ tissue culture flasks and incubated in a humidified mixture of 5% $CO_2$/95% air under atmospheric pressure. After reaching confluency, the cells were trypsinized and diluted to approximately $10^6$ cells/ml. For mRNA experiments, approximately $3 \times 10^6$ cells in 5 ml of medium were passaged to 25-cm$^2$ tissue culture flasks. Solutions of the chemicals dissolved in DMSO were added to the flasks so that the final concentration of DMSO in the medium was 0.5–1%. Cells were harvested and assayed for P450IA1 mRNA 18 hr after dosing.

**Treatment of animals.** Male Long-Evans rats (21 days old, 100 g) were obtained from Harlan Laboratories (Houston, TX) and housed two per cage in plastic cages with hardwood bedding. Rats were allowed free access to Purina certified rodent chow 5002 and water and were maintained on a diurnal cycle of 12 hr of light and 12 hr of darkness.

**Preparation of hepatic cytosol.** Rat hepatic cytosol was prepared according to the described procedures (31) and was stored in liquid nitrogen until used for binding studies. Male Long-Evans rats were sacrificed by cervical dislocation and the livers were perfused *in situ* with ice-cold HEDG buffer, via the inferior vena cava. Livers were homogenized in HEDG (3 ml/g of tissue) using a Brinkman Pt45/80 homogenizer. The homogenates were centrifuged at 10,000 × *g* for 20 min (2°), and the resulting supernatant was centrifuged at 105,000 × *g* for 1 hr (2°). The resulting clear supernatant was removed with a Pasteur pipette without disturbing the surface lipid layer or microsomal pellet. Protein concentrations were determined as described (32).

**P4501A1 mRNA analysis.** The *CYP1A1* cDNA probe was a gift from Dr. Alan Anderson (Laval University, Quebec City, Canada). The 0.9-kb fragment of *CYP1A1* was inserted in the *Pst*I site of pBR322. The plasmid pGMB1.1 was a gift from Dr. Don Cleveland (Johns Hopkins University) and carries the mouse β-tubulin cDNA cloned into the *Eco*RI site of pGEM-1. Plasmids were prepared by the alkaline lysis method (33). Digestion of the specific plasmids with the appropriate restriction enzyme yields a 0.9-kb *Pst*I fragment and a 1.3-kb *Eco*RI fragment, for detection of *CYP1A1* and β-tubulin mRNA, respectively. The desired fragments were isolated from 1% agarose gels by electroelution and were radiolabeled using T4 polynucleotide kinase and [γ-$^{32}$P]ATP before hybridization, as described previously (34). Cellular RNA from the cells in the different treatment groups was extracted, electrophoresed, and transferred to nylon membranes as described previously (23, 24). The membranes were initially probed for P4501A1 mRNA levels and then stripped and rehybridized for β-tubulin mRNA (23, 24). The intensities of the mRNA signals were individually quantitated with a Betagen Betascope 603 blot analyzer imaging system, and quantitation of P450IA1 mRNA was standardized against that of β-tubulin.

**Transformation and gel shift assay.** A complementary pair of oligonucleotides containing the sequence 5′-GATCTGGCTCTTCT-CACGCAACTCCG-3′ was synthesized, purified by polyacrylamide gel electrophoresis, annealed (35), and $^{32}$P-labeled at the 5′ end using T4 polynucleotide kinase and [γ-$^{32}$P]ATP (34). DNA binding was measured using a gel shift assay. Rat hepatic cytosol (16 mg/ml protein) was incubated for 2 hr at 20° with the desired chemicals such that the final concentration of DMSO was 0.2% (v/v). Higher concentrations of TCDD were used in the transformation assay, compared with the induction studies, because of the lower sensitivity of the former procedure. After incubation, 400 ng of poly[d(I-C)] in HEGD buffer were added and the cytosol was incubated for 15 min at 20°. After the addition of $^{32}$P-labeled DRE, the mixture was incubated for an additional 15 min at 20°. Reaction mixtures were loaded onto a 5% polyacrylamide gel (acrylamide/bisacrylamide, 30:0.8) and electrophoresed at 120 V for approximately 3 hr in 0.9 M Tris-borate, 2 mM EDTA, pH 8.0. Gels were dried and protein-DNA interactions were determined by imaging with a Betagen Betascope 603 blot analyzer. The amount of $^{32}$P-labeled DRE bound in the ligand-inducible complex was estimated by measuring the radioactivity of the specifically bound retarded band and subtracting the amount of radioactivity present at the same position in a non-ligand-treated lane.

**Kinetic binding assays.** The time-dependent dissociation rates for ligand-DRE complexes were measured at 4° after a 15-min incubation with $^{32}$P-DRE at 20° to form the transformed Ah receptor complex. A 100-fold excess of unlabeled DRE was added to the incubation mixture and aliquots were analyzed by the gel mobility assay and quantitated as described above.

**Expression plasmid and DNA transfection.** The plasmid pMCAT 5.12, obtained from Dr. James Whitlock, Jr. (Stanford University), contains a single DRE oligonucleotide that confers TCDD-inducible control upon the mouse mammary tumor virus long terminal repeat promoter and bacterial *CAT* gene. Plasmids were prepared by alkaline lysis and cesium chloride banding techniques (31).

H-4-II E cells were grown to approximately 80% confluency in 150-cm$^2$ tissue culture flasks as described previously. pMCAT 5.12 (120 μg) was added directly to 500 μl of 2× HEPES-buffered saline (40 mM HEPES, 0.28 M NaCl, 1.5 mM $Na_2HPO_4$, pH 7.05) containing 62 μl of 2 M $CaCl_2$. The solution was diluted to 1 ml with distilled water and the plasmid DNA was allowed to precipitate undisturbed for 30 min. Immediately before transfection, the α-MEM was removed from the tissue culture plates and replaced with 15 ml of α-MEM without fetal calf serum. Approximately 100 μl of DNA preparation containing 120 μg of pMCAT 5.12 were 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 with α-MEM twice, and allowed to grow for 12 hr. The cells were then harvested by trypsinization and plated into 25-cm$^2$ tissue culture flasks. After 8–12 hr, cells were treated with the desired chemicals.

**CAT assay.** After treatment with the desired chemicals for 24 hr, cells were harvested by manual scraping from the plate, centrifuged at 100 × *g* (2°) for 5 min, and resuspended in 20 μl of 0.25 M Tris·HCl (pH 7.8) in Eppendorf microfuge tubes. Cells were disrupted by exposure to three freeze-thaw cycles (1 min in acetone-dry ice and 1 min in a 37° water bath, respectively), the homogenate was vortexed for 15 sec, and the cell debris was removed by centrifugation at 10,000 × *g* (2°) for 5 min. The clear supernatant was extracted with a Hamilton syringe and placed into a fresh Eppendorf microfuge tube. 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 were placed into a fresh microfuge 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]chlor-

amphenicol (54 mCi/mmol, 20 μM) and acetyl-coenzyme A (4 mM). The reaction was allowed to continue at 37° for 30 min, with gentle mixing of samples at 10-min intervals. The reaction was terminated by the addition of 100 μl of ethyl acetate, followed by vortexing for 10 sec. The samples were centrifuged at 10,000 × *g* for 5 min, and the upper organic phase was removed and placed into a fresh Eppendorf microfuge tube. 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 thin layer chromatography plates. The samples were placed in thin layer chromatography chambers containing chloroform/methanol (95:5) and were retrieved when the solvent front was approximately 2–3 cm from the top of the plate. CAT activity was determined by quantitating acylated and nonacylated [$^{14}$C] chloramphenicol with a Betagen Betascope 603 imaging system.

**Statistical analysis.** The statistical differences between different groups were determined by analysis of variance and the levels of probability are noted ($p < 0.05$ or $p < 0.01$). The data are expressed as means ± standard errors for at least three determinations for each experiment.

## Results

The effects of different concentrations of αNF ($10^{-9}$ to $10^{-5}$ M) on the TCDD-induced transformation of the rat hepatic cytosolic receptor at 20°, as determined by gel mobility shift assays, are summarized in Table 1. The intensity of the retarded DRE complex obtained after incubation of cytosol with 1 nM TCDD alone was arbitrarily set at 100%, and the relative band intensities in the different treatment groups were compared with this value. Cotreatment of the cytosol with 1 nM TCDD plus 1 or 10 nM αNF did not affect the intensity of the TCDD-induced retarded band, whereas concentration-dependent decreases were observed when higher concentrations (100 nM or 1 μM) of αNF were used. In contrast, treatment of cytosol with 10 μM αNF alone or 10 μM αNF plus 1 nM TCDD gave retarded band intensities that were similar to those observed for 1 nM TCDD alone. The results illustrated in Figs. 1 and 2 show the concentration-dependent formation of the transformed rat cytosolic Ah receptor after incubation with different concentrations of αNF or TCDD alone. The results in Fig. 1 are expressed as a percentage of the retarded band intensities observed after incubation with 100 nM TCDD, and the results show that concentrations of 10 μM αNF and 1 nM TCDD were approximately equipotent as inducers of transformation of rat hepatic cytosol. The data also indicate that at higher concentrations of TCDD (10, 100, and 1000 nM) the levels of the transformed cytosolic Ah receptor also increased and saturation of this process occurred at 100 nM TCDD. Comparable saturation binding-transformation studies could not be carried out with αNF because of solubility problems associated with concentrations greater than 10 μM. The results in Fig. 3 illustrate the time-dependent dissociation of the transformed rat cytosol Ah receptor, liganded with either 20 nM TCDD or 10 μM αNF, from $^{32}$P-DRE at 4°. The data show that both complexes exhibited no significant dissociation from the $^{32}$P-DRE for the 90-min duration of the experiment.

The results in Figs. 4 and 5 summarize the concentration-dependent induction of *CYP1A1* mRNA levels by TCDD and αNF in rat hepatoma H-4-II E cells. The data (Fig. 4) are expressed as a percentage of the *CYP1A1* mRNA levels induced by 100 nM TCDD. αNF did not significantly induce *CYP1A1* mRNA levels at concentrations of 10, 100, or 1000 nM; however, at a concentration of 10 μM there was a sharp increase in *CYP1A1* mRNA levels, which were similar to those observed after treatment with 10 or 100 nM TCDD.

The functional activity of αNF as an Ah receptor agonist was also confirmed by determining the concentration-dependent induction of CAT activity by αNF in H-4-II E cells transiently transfected with the plasmid pMCAT 5.12, which contains a single DRE enhancer element upstream of the mouse mammary tumor virus promoter and the bacterial *CAT* gene. The results summarized in Table 2 and Fig. 6 also demonstrate that 10 and 100 nM αNF did not induce CAT activity; 1000 nM αNF caused minimal (but significant) induction of CAT activity and 10 μM αNF was more active than 1 nM TCDD as an inducer of CAT activity.

TABLE 1

**Effects of TCDD, αNF, and their combination on the transformation of the rat hepatic cytosolic receptor**

Rat hepatic cytosol was transformed for 2 hr at 20° with 1 nM TCDD or 1 nM TCDD plus αNF (1 nM to 10 μM). Transformed cytosol was then subjected to gel retardation analysis as described in Materials and Methods. The results are expressed as the means ± standard errors for at least four determinations for each treatment group.

| Treatment | DRE binding |
|---|---|
| | *% of response to 1 nM TCDD* |
| 1 nM *TCDD* | 100 ± 0 |
| 1 nM TCDD + 1 nM αNF | 101 ± 8.7[a] |
| 1 nM TCDD + 10 nM αNF | 94.1 ± 10.6[a] |
| 1 nM TCDD + 100 nM αNF | 38.4 ± 6.1[b,c] |
| 1 nM TCDD + 1 μM αNF | 15.9 ± 9.1[b,c] |
| 1 nM TCDD + 10 μM αNF | 123.3 ± 30.8[a] |
| 10 μM αNF | 142.7 ± 15.7[a] |

[a] Not statistically different ($p > 0.05$) from cytosol transformed with TCDD alone.
[b] Statistically lower ($p < 0.05$) than cytosol transformed with 1 nM TCDD plus 10 μM αNF.
[c] Statistically lower ($p < 0.05$) than cytosol transformed with 10 μM αNF alone.

Previous studies have shown that αNF antagonizes TCDD- and benzo[*a*]pyrene-induced *CYP1A1* gene expression in Ah-responsive mammalian cells in culture (23–26). Gasiewicz and Rucci (28) reported that αNF caused a concentration-dependent (1–1000 nM) inhibition of TCDD-induced transformation of rat hepatic cytosolic receptor after incubation for 2 hr at 20°. Comparable results were also obtained in the present study (Table 1; Fig. 1). It was also previously shown (28) that transformed cytosolic receptors liganded with the Ah receptor agonists TCDD, 2,3,7,8-TCDF, and βNF were relatively stable and cotreatment with 1 μM αNF did not decrease this stability. Moreover, in cotreatment studies the αNF-mediated inhibition of the formation of the transformed TCDD-Ah receptor complex was reversible after the addition of 2,3,7,8-TCDF, a strong Ah receptor agonist (28). It was hypothesized that αNF bound to the cytosolic Ah receptor complex but was unable to initiate the subsequent conformational or biochemical changes required for the transformation process and the formation of the DRE-binding complex (28).

Previous studies in this laboratory have demonstrated that both MCDF and αNF partially antagonize TCDD-induced *CYP1A1* gene expression in Ah-responsive mammalian cells (18, 20–26). Although there are some differences in the cellular disposition of these compounds, both MCDF and αNF competitively inhibited the saturation binding of [$^{3}$H]TCDD to the cytosolic Ah receptor. Moreover, like αNF (Table 1), MCDF also caused a concentration-dependent (20–200 nM) inhibition

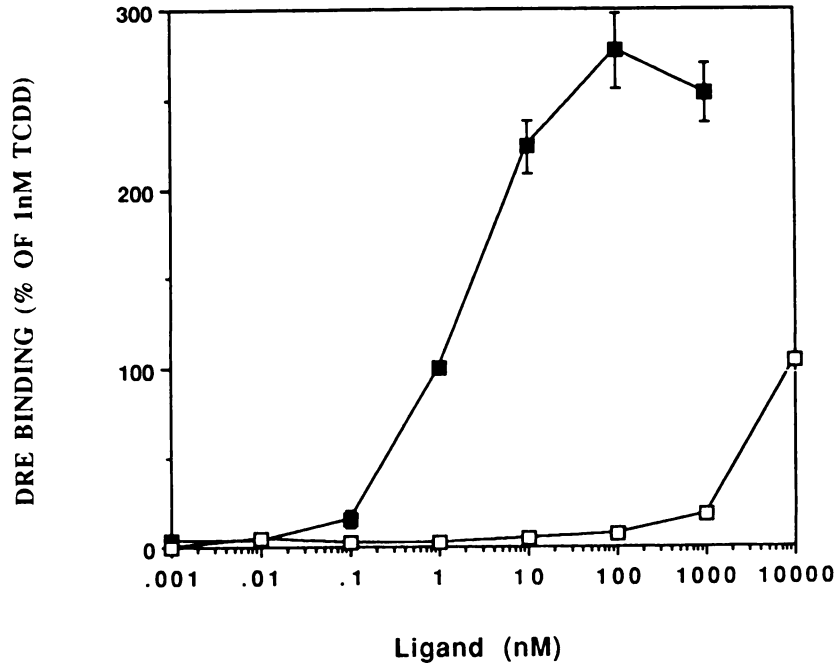


**Fig. 1.** Effects of different concentrations of TCDD (■) and αNF (□) on the transformation of rat cytosolic Ah receptor. The $^{32}$P-DRE was incubated with cytosolic extracts transformed with 0.001–1000 nM TCDD or 0.001–10,000 nM αNF. The data are expressed as a percentage of the response observed for transformation of cytosol incubated with 100 nM TCDD alone. Ah receptor-DRE interactions were determined by gel retardation analysis and the results are expressed as the means ± standard errors for at least three determinations for each concentration of TCDD/αNF.

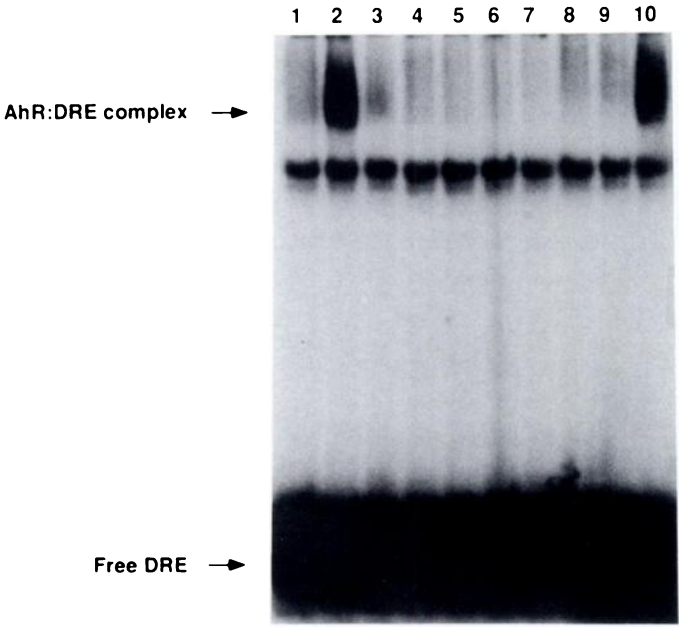


**Fig. 2.** Gel retardation analysis of transformed rat cytosolic Ah receptor from male Long Evans rats with a $^{32}$P-labeled consensus DRE. The $^{32}$P-DRE was incubated with cytosolic extracts transformed with DMSO (*lane 1*), 20 nM TCDD (*lane 2*), 0.001 nM αNF (*lane 3*), 0.01 nM αNF (*lane 4*), 0.1 nM αNF (*lane 5*), 1.0 nM αNF (*lane 6*), 10 nM αNF (*lane 7*), 100 nM αNF (*lane 8*), 1000 nM αNF (*lane 9*), or 10,000 nM αNF (*lane 10*). *Arrow*, specifically bound Ah receptor-DRE band. Incubation of the transformed receptor-$^{32}$P-DRE complex with a 50-fold excess of unlabeled DRE reduces the radioactivity only in the specifically labeled band (data not shown).

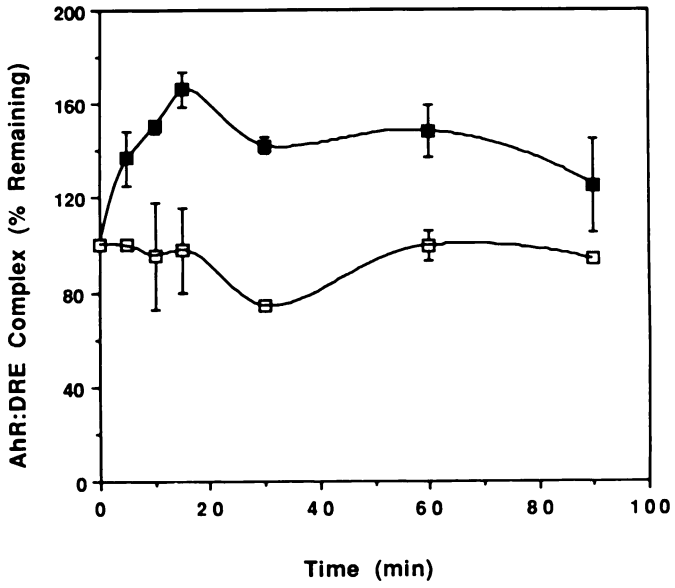


**Fig. 3.** Time-dependent dissociation of the transformed TCDD (■)- or αNF (□)-Ah receptor complexes from $^{32}$P-DRE at 4°. Cytosol was transformed with 20 nM TCDD or 10 μM αNF and incubated with a $^{32}$P-labeled DRE probe. The reaction mixtures were then incubated for various times with excess unlabeled DRE at 4°, and the data are expressed as a percentage of the response for specifically bound complex observed for TCDD or αNF at time zero. The percentage of Ah receptor-DRE complex remaining was determined by gel retardation analysis and the results are expressed as the means ± standard errors for at least three determinations at each time point.

of the TCDD-induced transformation of the rat cytosolic Ah receptor (data not shown). Previous studies (22) using an $^{125}$I-labeled analog of MCDF (i.e., $^{125}$I-MCDF) showed that this radioligand bound to the cytosolic Ah receptor to form a 9–10 S complex (untransformed); however, the apparent $B_{max}$ values derived from the saturation binding curve were only 1.5% of those obtained using [$^3$H]TCDD as the radioligand (22). Recent studies with 1,2,7,8-[$^3$H]TCDF, another weak Ah receptor agonist, also gave apparent $B_{max}$ values that were only 6% of those observed using [$^3$H]TCDD as the ligand (36). Thus, the direct binding studies with $^{125}$I-MCDF and 1,2,7,8-[$^3$H]TCDF show that both ligands form low levels of cytosolic Ah receptor complex and, in mammalian cancer cells treated with these congeners, low concentrations of their corresponding nuclear

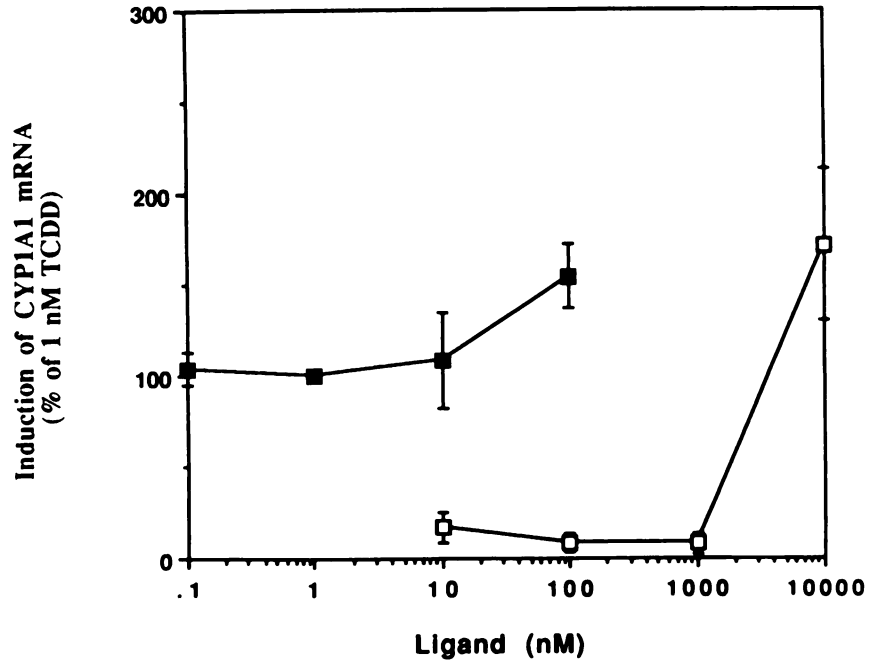


**Fig. 4.** Effect of different concentrations of TCDD (■) and αNF (□) on P4501A1 mRNA levels. The P4501A1 mRNA levels were determined by Northern analysis of mRNA extracted from the cells 24 hr after treatment with chemicals. The results are expressed as a percentage of the response observed for cells treated with 100 nM TCDD and represent the means ± standard errors for at least three determinations for each concentration of TCDD or αNF.

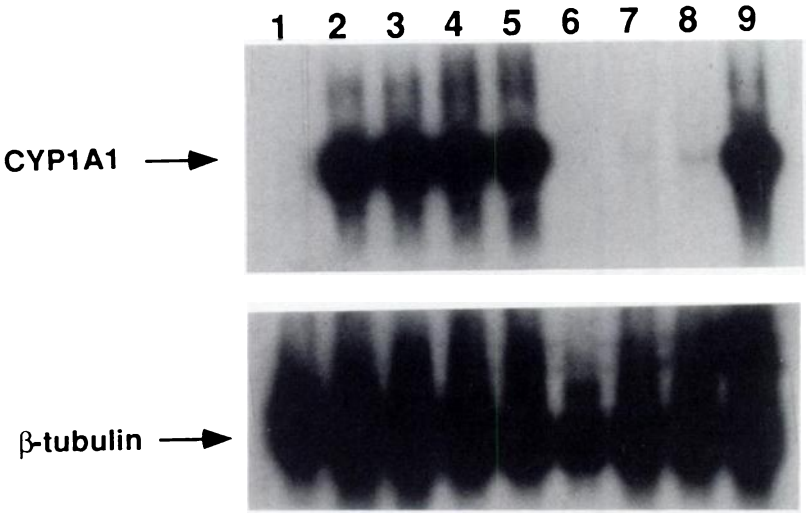


**Fig. 5.** Northern analysis of P4501A1 mRNA levels in rat hepatoma H-4-II E cells treated with DMSO (*lane 1*), 0.1 nM TCDD (*lane 2*), 1.0 nM TCDD (*lane 3*), 10 nM TCDD (*lane 4*), 100 nM TCDD (*lane 5*), 10 nM αNF (*lane 6*), 100 nM αNF (*lane 7*), 1000 nM αNF (*lane 8*), or 10,000 nM αNF (*lane 9*). The procedures used for RNA extraction and analysis are summarized in Materials and Methods.

TABLE 2

**Effects of TCDD and αNF on CAT activity in rat hepatoma H-4-II E cells transiently transfected with the Ah-responsive pMCAT 5.12 plasmid**

Cells were transfected with pMCAT 5.12, treated with chemicals for 24 hr, and then assayed for CAT activity as described in Materials and Methods.

| Treatment | CAT |
|---|---|
| | *% of maximum* |
| 1 nM TCDD | 100[a] |
| DMSO | 3 ± 3 |
| 10 nM αNF | 2 ± 2[b] |
| 100 nM αNF | 2 ± 1[b] |
| 10 μM αNF | 11 ± 3[a] |
| 100 μM αNF | 116 ± 10[a] |

[a] Statistically higher ($p < 0.01$) than cells treated with DMSO alone.
[b] Not statistically different ($p > 0.05$) from cells treated with DMSO alone.

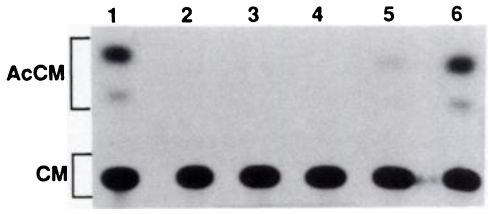


**Fig. 6.** Effects of different concentrations of αNF and 1 nM TCDD on the induction of CAT activity. H-4-II E cells were transfected with pMCAT 5.12 and treated with 1 nM TCDD (*lane 1*), DMSO (*lane 2*), 10 nM αNF (*lane 3*), 100 nM αNF (*lane 4*), 1000 nM αNF (*lane 5*), or 10 μM αNF (*lane 6*). Whole-cell extracts from cells were incubated with [$^{14}$C]chloramphenicol for 30 min at 37°. The substrate and products were extracted and subjected to thin layer chromatography as described in Materials and Methods. Chloramphenicol (*CM*) and acetylated chloramphenicol (*AcCM*) are noted in this figure.

Ah receptor complexes have been detected (22, 36). Kinetic analysis of the binding data for 1,2,7,8-[$^3$H]TCDF indicated that the Scatchard-derived low $B_{max}$ values may have been due to degradation of the cytosolic receptor and the calculated $K_d$

for 1,2,7,8-TCDF was significantly higher than the corresponding value obtained for TCDD (36). These observations are consistent with the low activity of these compounds as inducers of *CYP1A1* gene expression (37).

Because αNF exhibits some ligand-binding properties that are similar to those described for $^{125}$I-MCDF and 1,2,7,8-[$^{3}$H] TCDF, we hypothesize that the failure of this compound to transform rat cytosolic Ah receptor complexes is related to its weak Ah receptor agonist activity and not the result of the formation of complexes that fail to undergo transformation (28). The results summarized in Fig. 2 confirm that, at concentrations from 10 to 1000 nM, αNF does not form appreciable levels of the DRE-binding complex; in cotreatment studies with 1 nM TCDD, αNF (10–1000 nM) caused a concentration-dependent inhibition of the TCDD-induced transformation (Table 1), as previously reported by Gasiewicz and Rucci (28). However, if a higher concentration of αNF (10 μM) was used in the *in vitro* transformation assay, the intensity of the DRE-binding band was similar to that observed using 1 nM TCDD. Thus, like other weak Ah receptor agonists (35) αNF can induce the transformation of the rat cytosolic Ah receptor at high concentrations, and the mobility of the transformed band (Figs. 1 and 2) was similar to that observed for other Ah receptor ligands (38). Moreover, the results illustrated in Fig. 3 also demonstrate that at 4° there was essentially no dissociation of the transformed cytosolic Ah receptor, liganded with TCDD or αNF, from $^{32}$P-DRE.

The results of previous studies with αNF in transformed rodent cancer cell lines showed that at a concentration of 1000 nM only minimal induction of *CYP1A1* gene expression was observed and this correlated with low levels of DRE binding of nuclear extracts from these cells (23–26). Similar results were observed in this study (Table 2; Figs. 4–6); however, at an αNF concentration of 10 μM there was marked induction of *CYP1A1* mRNA levels and CAT activity in cells transfected with the TCDD-responsive plasmid pMCAT 5.12. Moreover, the CAT activity and *CYP1A1* mRNA levels induced by 10 μM αNF were similar to those observed with 1 nM TCDD. Thus, the concentration-dependent *in vitro* transformation of rat hepatic cytosol and the induction of *CYP1A1* gene expression by 10 μM αNF observed in this study suggest that αNF is both a partial Ah receptor agonist and an antagonist. The results also show that αNF can induce the transformation of the cytosolic Ah receptor and, therefore, the Ah receptor agonist activity of this compound is probably due to competition for ligand binding sites on the cytosolic Ah receptor.

**References**


1. Poland, A., and 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:**517–554 (1982).
2. Whitlock, J. P., Jr. The regulation of gene expression of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Pharmacol. Rev.* **39:**147–161 (1987).
3. Whitlock, J. P., Jr. Genetic and molecular aspects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin action. *Annu. Rev. Pharmacol. Toxicol.* **30:**251–277 (1990).
4. Safe, S. Comparative toxicology and mechanism of action of polychlorinated dibenzo-*p*-dioxins and dibenzofurans. *Annu. Rev. Pharmacol. Toxicol.* **26:**371–399 (1986).
5. Jones, P. B., L. K. Durrin, D. R. Galeazzi, and J. P. Whitlock, Jr. Control of cytochrome $P_1$-450 gene expression: analysis of a dioxin-responsive enhancer system. *Proc. Natl. Acad. Sci. USA* **83:**2802–2806 (1986).
6. Sogawa, K., A. Fujisawa-Sehara, M. Yamane, and Y. Fuji-Kuriyama. Location of regulatory elements responsible for drug induction in the rat cytochrome P-450c gene. *Proc. Natl. Acad. Sci. USA* **83:**8044–8048 (1986).
7. Denison, M. S., J. M. Fisher, and J. P. Whitlock, Jr. Inducible, receptor-dependent protein-DNA interactions at a dioxin-reponsive transcriptional enhancer. *Proc. Natl. Acad. Sci. USA* **85:**2528–2532 (1988).
8. Gonzalez, F. J., and D. W. Nebert. Autoregulation plus upstream positive and negative control regions associated with transcriptional activation of the mouse cytochrome $P_1$-450 gene. *Nucleic Acids Res.* **13:**7269–7288 (1985).
9. Ema, M., K. Sogawa, N. Watanabe, Y. Chujoh, N. Matsushita, O. Gotoh, Y. Funae, and Y. Fujii-Kuriyama. cDNA cloning and structure of the putative Ah receptor. *Biochem. Biophys. Res. Commun.* **184:**246–253 (1992).
10. Hoffman, E. C., H. Reyes, F.-F. Chu, F. Sander, L. H. Conley, B. A. Brooks, and O. Hankinson. Cloning of a factor required for activity of the Ah (dioxin) receptor. *Science (Washington D. C.)* **252:**954–958 (1991).
11. Reyes, H., S. Reisz-Porszasz, and O. Hankinson. Identification of the Ah receptor nuclear translocator protein (Arnt) as a component of the DNA binding form of the Ah receptor. *Science (Washington D. C.)* **256:**1193–1195 (1992).
12. Evans, R. M. The steroid and thyroid hormone superfamily. *Science (Washington D. C.)* **240:**889–895 (1988).
13. Bannister, R., D. Davis, T. Zacharewski, I. Tizard, and S. Safe. Aroclor 1254 as a 2,3,7,8-tetrachlorodibenzo-*p*-dioxin antagonist: effects on enzyme activity and immunotoxicity. *Toxicology* **46:**29–42 (1987).
14. Davis, D., and S. Safe. Immunosuppressive activities of polychlorinated biphenyls in C57BL/6 mice: structure-activity relationships as Ah receptor agonists and partial antagonists. *Toxicology* **63:**97–111 (1990).
15. Blank, J. A., A. N. Tucker, J. Sweatlock, T. A. Gasiewicz, and M. I. Luster. α-Naphthoflavone antagonism of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced murine lymphocyte ethoxyresofurin *O*-deethylase activity and immunosuppression. *Mol. Pharmacol.* **32:**168–172 (1987).
16. Luster, M. I., L. H. Hong, R. Osborne, J. A. Blank, G. Clark, M. T. Silver, G. A. Boorman, and W. F. Greenlee. 1-Amino-3,7,8-trichlorodibenzo-*p*-dioxin: a specific antagonist for TCDD-induced myelotoxicity. *Biochem. Biophys. Res. Commun.* **139:**747–756 (1986).
17. Keyes, B., J. Piskorska-Pliszczynska, and S. Safe. Polychlorinated dibenzofurans as 2,3,7,8-TCDD antagonists: *in vitro* inhibition of monooxygenase induction. *Toxicol. Lett.* **31:**151–158 (1986).
18. Astroff, B., T. Zacharewski, S. Safe, M. P. Arlotto, A. Parkinson, P. Thomas, and 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:**231–236 (1988).
19. Astroff, B., and S. Safe. 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).
20. Harris, M., T. Zacharewski, B. Astroff, and 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:**729–735 (1989).
21. Astroff, B., and S. Safe. 6-Substituted 1,3,8-trichlorodibenzofurans as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin antagonists in the rat: structure-activity relationships. *Toxicology* **59:**285–296 (1989).
22. Piskorska-Pliszczynska, J., B. Astroff, T. Zacharewski, M. Harris, R. Rosengren, V. Morrison, L. Safe, and S. Safe. Mechanism of action of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin antagonists: characterization of 6-[$^{125}$I]methyl-8-iodo-1,3-dichlorodibenzofuran-Ah receptor complexes. *Arch. Biochem. Biophys.* **284:**193–200 (1991).
23. Merchant, M., L. Arellano, and S. Safe. The mechanism of action of α-naphthoflavone as an inhibitor of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin induced *CYP1A1* gene expression. *Arch. Biochem. Biophys.* **281:**84–89 (1990).
24. Merchant, M., X. Wang, C. Kamps, R. Rosengren, V. Morrison, and S. Safe. Mechanism of benzo[*a*]pyrene-induced *CYP1A1* gene expression in mouse Hepa 1c1c7 cells: role of the nuclear 6 S and 4 S proteins. *Arch. Biochem. Biophys.* **292:**250–257 (1992).
25. Merchant, M., V. Morrison, M. Santostefano, and S. Safe. Mechanism of action of aryl hydrocarbon receptor antagonists: inhibition of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced *CYP1A1* gene expression. *Arch. Biochem. Biophys.* **298:**389–394 (1992).
26. Merchant, M., V. Krishnan, and S. Safe. Mechanism of action of α-naphthoflavone as an Ah receptor antagonist in MCF-7 human breast cancer cells. *Toxicol. Appl. Pharmacol.*, in press.
27. Zacharewski, T., M. Harris, L. Biegel, V. Morrison, M. Merchant, and 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:**311–318 (1992).
28. Gasiewicz, T. A., and G. Rucci. α-Naphthoflavone acts as an antagonist of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin by forming an inactive complex with the Ah receptor. *Mol. Pharmacol.* **40:**607–612 (1991).
29. Diamond, L., and H. V. Gelboin. *Alpha*-naphthoflavone: an inhibitor of hydrocarbon cytotoxicity and microsomal hydroxylase. *Science (Washington D. C.)* **166:**1023–1025 (1969).
30. Diamond, L., R. McFall, J. Miller, and H. V. Gelboin. The effects of two isomeric benzoflavones on aryl hydrocarbon hydroxylase and the toxicity and carcinogenicity of polycyclic hydrocarbons. *Cancer Res.* **32:**732–736 (1972).
31. Bandiera, S., S. Safe, and A. B. Okey. Binding of polychlorinated biphenyls classified as either phenobarbitone-, 3-methylcholanthrene-, or mixed-type inducers to cytosolic Ah receptor. *Chem. Biol. Interact.* **39:**259–277 (1982).
32. Bradford, M. M. A rapid and sensitive method for the quantitation of

microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
33. Sambrook, J., E. Fritsch, and T. Maniatis. *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989).
34. Maniatis, T., E. F. Fritsch, and J. Sambrook. *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 187–210 (1982).
35. Denison, M. S., and R. M. Deal. The binding of transformed aromatic hydrocarbon (Ah) receptor to its DNA recognition site is not affected by metal depletion. *Mol. Cell. Endocrinol.* **69**:51–57 (1990).
36. Rosengren, R., S. Safe, and N. J. Bunce. Kinetics of the association of several tritiated polychlorinated dibenzo-*p*-dioxin and dibenzofuran congeners with hepatic cytosolic Ah receptor from the Wistar rat. *Chem. Res. Toxicol.* **5**:376–382 (1992).
37. Zacharewski, T., M. Harris, and S. Safe. Induction of cytochrome P-450-dependent monooxygenase activities in rat hepatoma H-4II E cells in culture by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and related compounds: mechanistic studies using radiolabeled congeners. *Arch. Biochem. Biophys.* **272**:344–355 (1989).
38. Santostefano, M., J. Piskorska-Pliszczynska, V. Morrison, and S. Safe. Effects of ligand structure on the *in vitro* transformation of the rat cytosolic aryl hydrocarbon receptor. *Arch. Biochem. Biophys.* **297**:73–79 (1992).

**Send reprint requests to:** Stephen H. Safe, Department of Veterinary Physiology and Pharmacology, Texas Veterinary Medical Center, Texas A & M University, College Station, TX 77843–4466.