0026-895X/88/020231-06$02.00/0

MOLECULAR PHARMACOLOGY, 33:231-236

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

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Received September 21, 1987; Accepted November 9, 1987

SUMMARY

In addition to being one of the most toxic chemicals known, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is the most potent inducer of rat liver microsomal cytochrome P-4501A1 (P-450c). Previous studies have demonstrated that a high affinity, low capacity cytosolic receptor (the Ah receptor) mediates the activity of TCDD to induce cytochrome P-4501A1, which catalyzes benzo[*a*]pyrene hydroxylation [aryl hydrocarbon hydroxylase (AHH)] and 7-ethoxyresorufin *O*-dealkylation (EROD). The results of the present study indicate that 6-methyl-1,3,8-trichlorodibenzofuran (MCDF) effectively competes with [^{3}H]TCDD for binding to the Ah receptor in rat liver cytosol. The concentration of MCDF effecting 50% displacement of [^{3}H]TCDD was 4.9×10^{-8} M, which is ~50 times greater than the EC_{50} for unlabeled TCDD ($\sim 1 \times 10^{-9}$ M). However, in contrast to TCDD, MCDF was only a weak inducer of AHH and EROD activity in rat hepatoma H-4-II cells in culture. When co-incubated, MCDF diminished in a concentration-dependent manner the ability of TCDD to induce AHH and EROD activity *in vitro*. Treatment of rats with 20–200 μmol/kg MCDF *in vivo* had little or no effect on liver microsomal AHH and EROD activity, whereas treatment of rats with 16 nmol/kg TCDD caused a 6- and a 70-fold induction of AHH and EROD activity, respectively. When co-administered, MCDF diminished by ~50% the ability of TCDD to induce AHH and EROD activity *in vivo*. The partial antagonism produced by 50 μmol/kg MCDF could be partially overcome by doubling the dosage of TCDD from 16 to 32 nmol/kg. Immunochemical analysis of rat liver microsomes revealed that treatment of rats with 20–200 μmol/kg MCDF caused little or no induction of cytochromes P-4501A1 and P-4501A2 (P-450d), whereas these isozymes were induced 33- and 5-fold, respectively, in rats treated with 16 nmol/kg TCDD. When co-administered, MCDF diminished by ~50% the ability of TCDD to induce cytochrome P-4501A1 *in vivo*, which established that MCDF was not simply acting as an inhibitor of AHH and EROD activity. MCDF also antagonized the ability of TCDD to induce cytochrome P-4501A2, which suggests that the induction of both cytochromes P-4501A1 and P-4501A2 is regulated by the Ah receptor. These results indicate that MCDF binds with high affinity to the Ah receptor in rat liver cytosol and competitively blocks the binding of TCDD. However, MCDF is only a weak agonist and, as such, can antagonize the ability of TCDD to induce rat liver microsomal cytochromes P-4501A1 and P-4501A2 and associated monooxygenase activity. Its antagonistic properties may make MCDF a valuable probe to study the role of the Ah receptor in mediating the teratogenic, toxic, and other biochemical effects of TCDD and related compounds.

TCDD and several structurally related halogenated aryl hydrocarbons elicit a broad spectrum of toxic and biologic effects, which include teratogenicity and reproductive dysfunction, hepatotoxicity and porphyria, dermal toxicity, endocrine-related disorders, carcinogenicity, immunotoxicity, a wasting syndrome, and the induction of several enzymes including cytochrome P-450-dependent microsomal monooxygenases (reviewed in Refs. 1–5). TCDD is a potent inducer of liver microsomal benzo[*a*]pyrene hydroxylase (AHH) and EROD in rats and in certain *responsive* strains of mice (typified by C57BL/6 mice). In other *nonresponsive* strains of mice (typified by DBA/2 mice), the potency with which TCDD induces AHH and EROD is significantly reduced (6, 7). It has been proposed that the mechanism of enzyme induction involves initial binding of

The financial assistance of the National Institutes of Health (Grant ES-03843) and the Texas Agricultural Experiment Station is gratefully acknowledged. A. P. is the recipient of Research Career Development Award ES-00166 and is supported by Grant ES-03765 from the National Institutes of Health. M. P. A. is supported by Training Grant ES-07079 from the National Institutes of Health.

ABBREVIATIONS: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; AHH, aryl hydrocarbon hydroxylase; EROD, 7-ethoxyresorufin *O*-dealkylation; Ah, aryl hydrocarbon; PCB, polychlorinated biphenyl; MCDF, 6-methyl-1,3,8-trichlorodibenzofuran; Hepes, 4-(2-hydroxyethyl ether)-1-piperazineethanesulfonic acid; HAP, hydroxylapatite.

TCDD to a receptor protein in target cells. Studies with [^{3}H]TCDD revealed the presence of a high affinity, low capacity Ah receptor protein in hepatic cytosol from C57BL/6 mice, but not from DBA/2 mice (1, 2, 8–10). Several studies with responsive and nonresponsive mice and their offspring have demonstrated that AHH induction, porphyria, immunotoxicity, and teratogenicity all segregate with the *Ah* locus (1, 2, 6, 7, 11–14).

The Ah receptor has been identified in diverse animal species, tissues, and mammalian cell lines (8–10, 15–17). Poland *et al.* (18) have demonstrated that covalent modification of the C57BL/6 mouse hepatic receptor with a photoaffinity reagent gave a covalently labeled protein with an apparent molecular weight of 95,000 (estimated from its relative mobility during sodium dodecyl sulfate-polyacrylamide gel electrophoresis).

Recent studies in our laboratory have identified several compounds, namely, the PCB mixture, Aroclor 1254, and 1,3,6,8- and 2,4,6,8-tetrachlorodibenzofuran, which partially antagonize one or more Ah receptor-mediated responses (i.e., teratogenicity, immunotoxicity, and AHH induction) in mammalian cells in culture or in C57BL/6 mice (19–21). These TCDD antagonists typically exhibit weak agonist activity ($<10^{-5}$ times as active as TCDD) and moderately high Ah receptor-binding affinities, which suggests that these weak agonists act as partial antagonists by competing with TCDD for binding to the Ah receptor. A similar mechanism of action has been proposed for 1-amino-3,7,8-trichlorodibenzo-*p*-dioxin, which antagonizes the immunotoxic effects of TCDD (22).

This study describes the synthesis of MCDF and demonstrates MCDF to be an effective antagonist of the TCDD-mediated induction of cytochrome P-450 isozymes and associated monooxygenase activity in male rats and in rat hepatoma H-4-II E cells in culture.

Materials and Methods

Chemicals and biochemicals. 2-Methyl-4-chlorophenol, isoamyl nitrite, and 2,4,6-trichloroaniline were purchased from Aldrich Chemical Co. (Milwaukee, WI). [^{3}H]TCDD, ethoxyresorufin, and 2,3,7,8-tetrachlorodibenzofuran were synthesized in our laboratories, with purities >99%. Benzo[*a*]pyrene, NADP, NADPH, D-glucose 6-phosphate, D-glucose-6-phosphate dehydrogenase, rhodamine B, bovine serum albumin, and Hepes were purchased from Sigma Chemical Co. (St. Louis, MO). HAP was purchased from Bio-Rad Laboratories (La Jolla, CA).

Synthesis and characterization of MCDF. MCDF was synthesized according to the scheme in Fig. 1. 2-Methyl-4-chlorophenol (20 g) and 2,4,6-trichloroaniline (5 g) were mixed and heated with stirring to 120°; isoamyl nitrite (6 ml) was added dropwise over 30 min and the mixture was stirred for 18 hr at 120°. The excess phenol was removed by evaporation, and the residue was adsorbed on silica gel and added to the top of a silica gel column packed with petroleum spirit/diethyl ether (3:7). The column was eluted with 500 ml of the above solvent, the solvent was evaporated, and the residue was redissolved in dimethyl sulfoxide. Anhydrous potassium carbonate (500 mg) was added and the mixture, which was stirred for 2 hr at 190°, was adsorbed on silica gel. After the solvent was evaporated, the silica gel was added to the top of a silica gel column packed and equilibrated with petroleum spirit. MCDF was eluted from the column with petroleum spirit (500 ml) and the residue was crystallized from anisole/methanol (9:1). The product (280 mg) was >99% pure as determined by gas chromatographic analysis, and a molecular weight (M^+) of 284 was confirmed with a VG 12000 quadrupole mass chromatograph coupled to a Hewlett-Packard 500 gas chromatograph. The 220-MHz proton magnetic resonance spectrum (in deuterochloroform) was determined with a Varian XL200 spectrometer and gave (in $CDCl_3$): 8.07 (H-9, d, J = 1.6 Hz) 7.48, 7.33 (H-1/H-3, d, J = 1.6 Hz); 7.29, 7.29 ppm (H-7, m).

Animal treatment, isolation of microsomes, and monooxygenases enzyme assays. Immature, male Long Evans rats (~100 g body weight) were purchased from Timco Breeding Laboratories (Houston, TX). The animals were housed in stainless steel cages, allowed free access to water and Purina Rat Chow 5002, and maintained on a 12-hr light cycle. MCDF and TCDD were dissolved in corn oil and administered individually or together to rats by intraperitoneal injection (5 ml/kg). Four rats were used in each treatment group, and corn oil-treated animals (5 ml/kg) served as controls. Three days after treatment, the animals were killed by cervical dislocation and liver microsomes were prepared as described (22). Microsomal AHH and EROD activity was measured by the fluorometric methods of Nebert and Gelboin (23), and of Pohl and Fouts (24), respectively. Protein concentration was determined by the method of Lowry *et al.* (25), with bovine serum albumin as standard. The concentration of cytochrome P-450 was determined by the method of Omura and Sato (26) from the CO difference spectrum of dithionite-reduced microsomes based on an extinction coefficient of 91 mM^{-1} cm^{-1}. Spectra were recorded on an Aminco DW-2A spectrophotometer.

Purification of antigens and immunochemical quantitation assays. Liver microsomal cytochrome P-4501A1 was purified to homogeneity from Aroclor 1254-treated rats, whereas cytochrome P-4501A2 was purified from isosafrole-treated rats, as described previously (27, 28). Antibodies to cytochromes P-4501A1 and P-4501A2 were raised in rabbits. The purification of the immunoglobulin fraction from each antiserum included absorption chromatography with appropriate heterologous antigens covalently bound to Sepharose 4B, as previously described (29). The concentrations of cytochromes P-4501A1 and P-4501A2 were determined by single radial immunodiffusion (29, 30).[1]

[1] According to a recently recommended nomenclature system for cytochromes P-450 (31), rat cytochromes P-450c and P-450d are encoded by genes *IA1* and IA2, respectively.

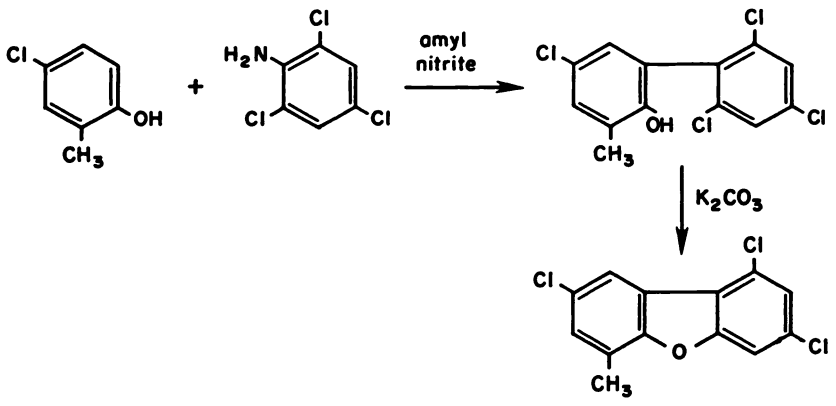

Fig. 1. Synthesis of MCDF.

Receptor binding assays. The EC_{50} for the competitive displacement of [^{3}H]TCDD from hepatic cytosol (3–5 mg of protein/ml) by MCDF was determined by the HAP procedure of Gasiewicz and Neal (32) as described by Bannister *et al.* (21). The same procedure was used determine the receptor binding of different concentrations of [^{3}H]TCDD in the presence of several concentrations of MCDF. Sucrose density gradient analysis of [^{3}H]TCDD-receptor complexes in the presence of different MCDF concentrations was determined as described (21).

Enzyme induction in cultured cells. Rat hepatoma H-4-II E cells were kindly supplied by Dr. J. Bradlaw, Food and Drug Administration (Washington, D. C.). The culture conditions, enzyme induction experiments, and methods used for the AHH and EROD assays were carried out as previously described (21).

Results

MCDF caused a concentration-dependent displacement of [^{3}H]TCDD from rat hepatic cytosolic receptor protein as determined by a sucrose density gradient (Fig. 2) and an HAP assay procedure. Using the HAP procedure (1 nM [^{3}H]TCDD, 3–5 mg of protein/ml of cytosol), the concentration of MCDF that displaced 50% of the bound TCDD was 4.9×10^{-8} M. The EC_{50} value is about 50 times higher than that obtained for unlabeled TCDD (1.0×10^{-9} M). Double reciprocal (Lineweaver-Burke) plots of the [^{3}H]TCDD receptor-binding data in the presence of different concentrations of MCDF by the HAP assay method are shown in Fig. 3. The plots were all linear and intercepted on the *y* axis. These data indicate that MCDF acts as a competitive inhibitor of the interaction between [^{3}H]TCDD and the rat liver receptor protein.

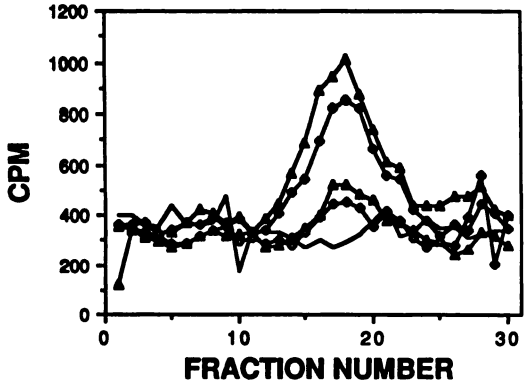

Fig. 2. Sucrose density gradient profiles of the [^{3}H]TCDD rat liver receptor complex in the presence of 0 (△), 5- (■), 10- (▲), and 100- (◇) fold excess of MCDF and a 300-fold excess of 2,3,7,8-tetrachlorodibenzofuran (——) (for complete displacement of the radioligand). The assay procedure was determined as described (19, 21).

The ability of TCDD, MCDF, and a combination of TCDD plus MCDF to induce AHH and EROD in rat hepatoma H-4-II E cells is summarized in Table 1. The EC_{50} values for the induction of AHH and EROD by TCDD in these cells were 9.8×10^{-11} and 1.9×10^{-10} M, respectively, and 80–100% maximal induction was observed at concentrations of TCDD between 7.75×10^{-10} and 10^{-8} M. In contrast, MCDF caused a concentration-dependent induction of AHH and EROD at concentrations of 10^{-7} and 10^{-6} M. At an MCDF concentration of 10^{-5} M, the induction of the monooxygenases was decreased due to cytotoxicity. Treatment of the cells with both TCDD (10^{-8} or 7.75×10^{-10} M) plus MCDF (10^{-6} and 10^{-7} M) resulted in significant inhibition of the induction of AHH and EROD by both concentrations of TCDD.

The ability of TCDD, MCDF, and a combination of both TCDD plus MCDF to induce rat liver microsomal AHH and EROD is summarized in Table 2. MCDF did not significantly induce hepatic microsomal AHH at dosages of 20, 50, and 200 μmol/kg, whereas a 4-fold induction of EROD was observed in rats administered the highest dose of MCDF. In contrast, treatment of rats with 16 nmol/kg TCDD resulted in a 6- and a 70-fold induction of AHH and EROD, respectively. Based on previous studies, this dosage of TCDD was estimated to result in submaximal induction of EROD activity (3). Treatment of the rats with both MCDF (20, 50, or 200 μmol/kg) and TCDD (16 nmol/kg) resulted in significant antagonism of the TCDD-mediated induction of AHH and EROD. Moreover, the partial antagonism by MCDF at a dosage of 50 μmol/kg could be partially overcome by treatment of rats with a higher dosage of TCDD (32 nmol/kg).

Table 3 summarizes the effects of treating rats with TCDD, MCDF, or both TCDD and MCDF on the levels of liver microsomal cytochromes P-4501A1 and P-4501A2. TCDD (16 nmol/kg) treatment resulted in a 33- and a 5-fold induction of cytochromes P-4501A1 and P-4501A2, respectively. In contrast, treatment of rats with MCDF (at doses of 20, 50, and 200 μmol/kg) did not significantly induce either cytochrome P-450 isozyme. Treatment of the animals with both TCDD (16 nmol/kg) and MCDF (20, 50, and 200 μmol/kg) resulted in significant inhibition of the TCDD-mediated induction of both cytochromes P-4501A1 and P-4501A2.

Discussion

Receptor antagonists can be effectively used as probes for understanding the mechanism of receptor-mediated processes.

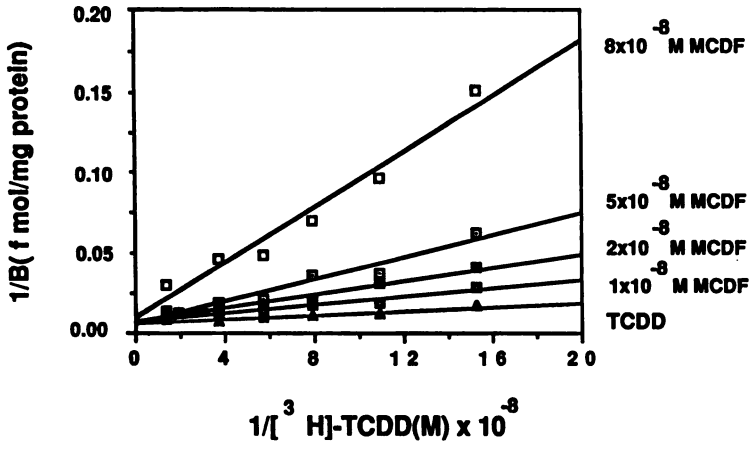

Fig. 3. Double reciprocal plot of the saturation binding of [^{3}H]TCDD in the presence of different concentrations of MCDF (8.0×10^{-8}, 2.0×10^{-8}, and 1.0×10^{-8} M). The linear correlation coefficients were >0.99 for all plots. Binding of [^{3}H]TCDD to the rat liver cytosolic receptor was determined by an HAP chromatographic procedure (21, 32).

TABLE 1

Effects of TCDD, MCDF, and TCDD plus MCDF as inducers of AHH and EROD in rat hepatoma H-4-II E cells in culture

Inducer	Concentration	EROD	AHH
	M	pmol/mg/min	
Solvent		1.20 ± 0.40	0
MCDF	10^{-7}	26.7 ± 1.64	16.7 ± 4.70
MCDF	10^{-6}	31.5 ± 1.08	33.9 ± 3.05
MCDF	10^{-5} [a]	14.4 ± 0.64	19.8 ± 2.57
TCDD	low conc.[b]	77.0 ± 3.11	84.1 ± 12.5
TCDD plus MCDF	10^{-7}	39.1 ± 4.44[c]	35.6 ± 3.32[c]
TCDD plus MCDF	10^{-6}	16.8 ± 0.43[c]	16.5 ± 6.18[c]
TCDD plus MCDF	10^{-5} [a]	14.4 ± 1.16[c]	12.7 ± 7.40[c]
TCDD	high conc.[d]	91.7 ± 2.53	109 ± 9.85
TCDD plus MCDF	10^{-7}	47.6 ± 3.41[e]	56.3 ± 4.61[e]
TCDD plus MCDF	10^{-6}	18.0 ± 0.84[e]	15.9 ± 1.99[e]
TCDD plus MCDF	10^{-5} [a]	14.8 ± 0.62[e]	15.9 ± 1.81[e]

[a] Cytotoxicity was observed.
[b] 7.75×10^{-10} M.
[c] Significantly lower ($p < 0.01$) than observed after treatment of the cells with TCDD at a concentration of 7.75×10^{-10} M.
[d] 1×10^{-8} M.
[e] Significantly lower ($p < 0.01$) than observed after treatment of the cells with TCDD at a concentration of 1×10^{-8} M.

TABLE 2

Induction of liver microsomal AHH and EROD in rats administered TCDD, MCDF, or both

Values are the mean ± standard deviation of four determinations.

Treatment	Dose	EROD	AHH
	μmol/kg	pmol/mg protein/min	
Corn oil		53 ± 22	315 ± 36
MCDF	20	100 ± 40	337 ± 53
MCDF	50	132 ± 16	337 ± 7
MCDF	200	230 ± 45	373 ± 47
TCDD	low dose[a]	3702 ± 410	1921 ± 100
TCDD plus MCDF	low dose[a] 20	1729 ± 450[b]	1208 ± 117[b]
TCDD plus MCDF	low dose[a] 50	1481 ± 361[b]	1042 ± 13[b]
TCDD plus MCDF	low dose[a] 200	1612 ± 600[b]	1007 ± 270[b]
TCDD	high dose[a]	4564 ± 340	3013 ± 318
TCDD plus MCDF	high dose[a] 50	3369 ± 252	2554 ± 233

[a] The low and high doses of TCDD were 16 and 32 nmol/kg, respectively.
[b] Significantly lower ($p < 0.01$) than values observed for induction by TCDD (16 nmol/kg) alone.

TABLE 3

Induction of liver microsomal cytochrome P-450 and P-450 isozymes in rats administered TCDD, MCDF, or both

Values are the mean ± standard deviation of four determinations.

Treatment[a]	Cytochrome P-450		
	Total	P-4501A1	P-4501A2
	nmol/mg		
Corn oil	0.885 ± 0.084	0.01 ± 0	0.04 ± 0.01
MCDF (20)	0.943 ± 0.150	0.01 ± 0	0.04 ± 0.01
MCDF (50)	0.990 ± 0.144	0.01 ± 0	0.04 ± 0.01
MCDF (200)	0.973 ± 0.115	0.01 ± 0	0.04 ± 0.01
TCDD	1.45 ± 0.046	0.33 ± 0.03	0.20 ± 0.03
TCDD plus MCDF (20)	1.27 ± 0.081	0.15 ± 0.02[b]	0.09 ± 0.03[b]
TCDD plus MCDF (50)	1.15 ± 0.158	0.16 ± 0.03[b]	0.06 ± 0.03[b]
TCDD plus MCDF (200)	1.32 ± 0.121	0.16 ± 0.02[b]	0.04 ± 0.02[b]

[a] Rats treated with TCDD received 16 nmol/kg. MCDF was administered at dosages of 20, 50, or 200 μmol/kg as indicated in parentheses.
[b] Significantly lower ($p < 0.01$) than values observed for induction by TCDD (0.016 μmol/kg) alone.

For example, the triphenyl ethylene estrogen receptor antagonists have been widely utilized to investigate the mechanism of estrogen action (23). Research in our laboratory on the development and identification of Ah receptor antagonists or partial antagonists has focused on PCBs, dibenzofuran, and dibenzo-*p*-dioxin congeners and mixtures which exhibit moderate Ah receptor-binding affinities but are weak agonists for one of the most sensitive and well characterized Ah receptor-mediated responses, namely, the induction of the cytochrome P-450-dependent monooxygenases, AHH and EROD (19–21). Preliminary screening studies with rat hepatoma H-4-II E cells in culture have shown that the commercial PCB mixture, Aroclor 1254, and 1,3,6,8- and 2,4,6,8-tetrachlorodibenzofuran antagonized the induction of AHH and EROD by TCDD (19, 21). Aroclor 1254 also antagonized the action of TCDD as a teratogen, as an immunotoxin, and as an inducer of liver microsomal AHH and EROD in C57BL/6 mice (20, 21). 1,3,6,8-Tetrachlorodibenzofuran also exhibited Ah receptor antagonist properties in the mouse (34); however, neither Aroclor 1254 nor 1,3,6,8-tetrachlorodibenzofuran was active as antagonists in the rat (data not shown).

MCDF is a methyl-substituted analog of 1,3,6,8-tetrachlorodibenzofuran that exhibits relatively high competitive Ah receptor-binding affinity ($EC_{50} = 4.9 \times 10^{-8}$ M by the HAP assay; Fig. 3) and low agonist activity for the induction of AHH or EROD in rat hepatoma H-4-II E cells in culture (Table 1). Moreover, the results of treatment with both TCDD and MCDF demonstrated that MCDF antagonizes the induction of AHH and EROD by TCDD in rat hepatoma cells. MCDF is a weak inducer of liver microsomal EROD in the rat but has no significant effect on AHH activity. However, treatment of the animals with both TCDD (16 nmol/kg) and MCDF (20, 50, or 200 μmol/kg) resulted in a >50% inhibition of the TCDD-mediated induction of EROD activity at all dosages of MCDF, and comparable results were observed for the induction of AHH activity. This antagonism by MCDF could be overcome, in part, by doubling the dose of TCDD, which suggests that MCDF does not simply act as an inhibitor of AHH and EROD activity.

In rat liver, cytochromes P-4501A1 and P-4501A2 are the major isozymes induced by TCDD (29). These isozymes are also co-inducible by several other xenobiotics, including 3-methylcholanthrene, various PCBs and polybrominated biphenyls, isosafrole, β-naphthoflavone, and phenothiazine (29, 30, 35). Cytochromes P-4501A1 and P-4501A2 possess high regions of primary amino acid sequence homology and are immunochemically related to each other (29, 36–39). Extensive structure-activity studies with PCBs and polybrominated biphenyls as inducers of AHH, EROD, cytochromes P-4501A1 and P-4501A2, and as ligands for the Ah receptor (30, 35, 40) support a role for the Ah receptor in mediating the induction of both cytochrome P-450 isozymes. The results summarized in Table 3 confirm that TCDD co-induces cytochromes P-4501A1 and P-4501A2, whereas MCDF was inactive as an inducer of these hemoproteins at dosages of 20, 50, and 200 μmol/kg. Treatment of rats with both TCDD and MCDF resulted in antagonism of the TCDD-mediated induction of cytochromes P-4501A1 and P-4501A2. At the highest concentration of MCDF (200 μmol/kg) there was complete inhibition of the induction of cytochrome P-4501A2. The ability of MCDF to antagonize the activity of TCDD to induce immunochemically reactive cytochromes P-4501A1 and P-4501A2 established

that MCDF was not simply acting as an inhibitor of AHH and EROD activity.

Two groups have reported the noncoordinate regulation of the cytochrome P-4501A1 and P-4501A2 mRNAs in rats (41, 42), whereas Hardwick *et al.* (43) have concluded that both mRNAs are coordinately regulated. The differences in these observations have been discussed (41) and have been attributed to several factors including the use of different chemical inducers and different methods for measuring P-450 mRNA levels. Comparable studies in mice support the role of the Ah receptor in the induction of the corresponding murine cytochrome P-450 mRNAs by aromatic hydrocarbons (44), and it has been suggested (41) that noncoordinate regulation of the rat mRNAs does not exclude a role for the Ah receptor in this process. The results obtained in this study with MCDF provide further support for the role of the Ah receptor in the induction of the final mRNA translation products, namely, cytochromes P-4501A1 and P-4501A2. Double reciprocal plot analysis of the competitive binding of MCDF and [^{3}H]TCDD (Fig. 3) suggests that MCDF acts as a competitive inhibitor that can decrease binding of the agonist (TCDD) with the Ah receptor. The identification of MCDF as an Ah receptor antagonist will facilitate future studies on the role of the Ah receptor in mediating the teratogenic, toxic, and other biochemical effects of TCDD and related compounds. Such studies are currently in progress.

References

1. Poland, A., W. F. Greenlee, and A. S. Kende. Studies on the mechanism of action of the chlorinated dibenzo-*p*-dioxins and related compounds. *Ann. N. Y. Acad. Sci.* **320**:214–230 (1979).
2. Poland, A., and J. C. Knutson. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin and related halogenated aromatic hydrocarbons: examination of the mechanism of toxicity. *Annu. Rev. Pharmacol. Toxicol.* **22**:517–554 (1982).
3. Safe, S. H. Comparative toxicology and mechanism of action of polychlorinated dibenzo-*p*-dioxins and dibenzofurans. *Annu. Rev. Pharmacol. Toxicol.* **26**:371–399 (1986).
4. Safe, S. Polychlorinated biphenyls (PCBs) and polybrominated biphenyls (PBBs): biochemistry, toxicology and mechanism of action. *CRC Crit. Rev. Toxicol.* **12**:319–395 (1984).
5. Whitlock, J. P. The regulation of gene expression by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Pharmacol. Rev.* **39**:147–161 (1987).
6. Poland, A. P., E. Glover, J. R. Robinson, and D. W. Nebert. Genetic expression of aryl hydrocarbon hydroxylase activity. Induction of monooxygenase activities and cytochrome P_1-450 formation by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in mice genetically "nonresponsive" to other aromatic hydrocarbons. *J. Biol. Chem.* **249**:5599–5606 (1974).
7. Poland, A., and E. Glover. Genetic expression of aryl hydrocarbon hydroxylase by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin: evidence for a receptor mutation in genetically non-responsive mice. *Mol. Pharmacol.* **11**:389–398 (1975).
8. Poland, A., E. Glover, and A. S. Kende. Stereospecific, high affinity binding of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin by hepatic cytosol. *J. Biol. Chem.* **251**:4936–4946 (1976).
9. Okey, A. B., G. P. Bondy, M. E. Mason, G. S. Kahl, H. J. Eisen, T. M. Guenthner, and D. W. Nebert. Regulatory gene product of the *Ah* locus. Characterization of the cytosolic inducer-receptor complex and evidence for its nuclear translocation. *J. Biol. Chem.* **254**:11636–11648 (1979).
10. Mason, M., and A. B. Okey. Cytosolic and nuclear binding of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin to the Ah receptor in extra-hepatic tissues of rats and mice. *Eur. J. Biochem.* **123**:209–215 (1982).
11. Nebert, D. W., and N. M. Jensen. The *Ah* locus: genetic regulation of the metabolism of carcinogens, drugs and other environmental chemicals by cytochrome P-450 mediated monooxygenases. *CRC Crit. Rev. Biochem.* **6**:401–437 (1979).
12. Poland, A., and E. Glover. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin: segregation of toxicity with the *Ah* locus. *Mol. Pharmacol.* **17**:86–94 (1980).
13. Jones, K. G., and G. D. Sweeney. Dependence of the porphyrinogenic effect of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin upon inheritance of aryl hydrocarbon hydroxylase responsiveness. *Toxicol. Appl. Pharmacol.* **53**:42–49 (1980).
14. Nagarkatti, P., G. D. Sweeney, J. Gauldie, and D. A. Clark. Sensitivity to suppression of cytotoxic T cell generation by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is dependent on the *Ah* genotype of the murine host. *Toxicol. Appl. Pharmacol.* **72**:169–176 (1984).
15. Manchester, D. K., S. K. Gordon, C. L. Golas, E. A. Roberts, and A. B. Okey. Ah receptor in human placenta: stabilization by molybdate and characterization of binding of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, 3-methylcholanthrene, and benzo(*a*)pyrene. *Cancer Res.* **47**:4861–4868 (1987).
16. Gasiewicz, T. A., and G. Rucci. Cytosolic receptor for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Evidence for a homologous nature among various mammalian species. *Mol. Pharmacol.* **26**:90–98 (1984).
17. Okey, A. B., G. P. Bondy, M. E. Mason, D. W. Nebert, C. J. Forster-Gibson, J. Muncan, and M. J. Dufresne. Temperature-dependent cytosol-to-nucleus translocation of the Ah receptor for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in continuous cell culture lines. *J. Biol. Chem.* **255**:11415–11422 (1980).
18. Poland, A., E. Glover, F. H. Ebettino, and A. S. Kende. Photoaffinity labelling of the Ah receptor. *J. Biol. Chem.* **261**:6352–6365 (1986).
19. Keys, 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).
20. Haake, J. M., S. Safe, K. Mayura, and T. D. Phillips. Aroclor 1254 as an antagonist of the teratogenicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Toxicol. Lett.* **38**:299–305 (1987).
21. 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 induction and immunotoxicity. *Toxicology* **46**:29–42 (1987) .
22. Luster, M. I., L. I. 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).
23. Nebert, D. W., and H. V. Gelboin. Substrate-inducible microsomal aryl hydroxylase in mammalian cell culture: assay and properties of induced enzyme. *J. Biol. Chem.* **242**:6242–6249 (1968).
24. Pohl, R. J., and J. R. Fouts. A rapid method for assaying the metabolism of 7-ethoxyresorufin by microsomal subcellular fractions. *Anal. Biochem.* **107**:150–155 (1980).
25. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
26. Omura, T., and R. Sato. The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its haemoprotein nature. *J. Biol. Chem.* **239**:2370–2378 (1964).
27. Ryan, D. E., P. E. Thomas, and W. Levin. Hepatic microsomal cytochrome P-450 from rats treated with isosafrole: purification and characterization of four enzymic forms. *J. Biol. Chem.* **255**:7941–9955 (1980).
28. Ryan, D. E., P. E. Thomas, and W. Levin. Purification and characterization of a minor form of hepatic microsomal cytochrome P-450 from rats treated with polychlorinated biphenyls. *Arch. Biochem. Biophys.* **216**:272–288 (1982).
29. Thomas, P. E., L. M. Reik, D. E. Ryan, and W. Levin. Induction of two immunochemically related rat liver cytochrome P-450 isozymes, cytochrome P-450c and P-450d by structurally diverse xenobiotics. *J. Biol. Chem.* **258**:4590–4598 (1983).
30. Parkinson, A., S. Safe, L. Robertson, P. E. Thomas, D. E. Ryan, L. M. Reik, and W. Levin. Immunochemical quantitation of cytochrome P-450 isozymes and epoxide hydrolase in liver microsomes from polychlorinated and polybrominated biphenyls: a study of structure-activity relationships. *J. Biol. Chem.* **258**:5967–5976 (1983).
31. Nebert, D. W., M. Adesnik, M. J. Coon, R. W. Estabrook, F. J. Gonzalez, F. P. Guengerich, I. C. Gunsalus, E. F. Johnson, B. Kemper, W. Levin, I. R. Phillips, R. Sato, and M. R. Waterman. The P-450 gene superfamily: recommended nomenclature. *DNA* **6**:1–11 (1987).
32. Gasiewicz, T. A., and R. A. Neal. The examination and quantitation of tissue cytosolic receptors for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin using hydroxylapatite. *Anal. Biochem.* **124**:1–11 (1982).
33. Furr, B. J. A., and V. C. Jordan. The pharmacology and clinical uses of tamoxifen. *Pharmacol. Ther.* **25**:187–205 (1984).
34. Bannister, R., and S. Safe. The effects of receptor antagonists on the AHH induction activity of 2,3,7,8-TCDD in C57BL/6 and DBA/2 mice. Proceedings, Dioxin '86 Symposium, Fukuoka, Japan. *Chemosphere* **16**:1739–1742 (1987).
35. Dannan, G. A., F. P. Guengerich, L. S. Kaminsky, and S. D. Aust. Regulation of cytochrome P-450. Immunochemical quantitation of eight isozymes in liver microsomes of rats treated with polybrominated biphenyl congeners. *J. Biol. Chem.* **258**:1282–1288 (1983).
36. Reik, L. M., W. Levin, D. E. Ryan, and P. E. Thomas. Immunochemical relatedness of rat hepatic microsomal cytochromes P-450c and P-450d. *J. Biol. Chem.* **257**:3950–3957 (1982).
37. Thomas, P. E., L. M. Reik, D. E. Ryan, and W. Levin. Characterization of nine monoclonal antibodies against the rat hepatic cytochrome P-450c. *J. Biol. Chem.* **259**:3890–3899 (1984).
38. Kawajiri, K., O. Gotoh, K. Sogawa, Y. Tagashiri, M. Muromatsu, and Y. Fujii-Kuriyama. Coding nucleotide sequence of 3-methylcholanthrene inducible cytochrome P-450d cDNA from rat liver. *Proc. Natl. Acad. Sci. USA* **81**:1649–1653 (1984).
39. Sogawa, K., O. Gotoh, K. Kawajiri, and Y. Fujii-Kuriyama. Distinct organization of methylcholanthrene- and phenobarbital-inducible cytochrome P-450 genes in the rat. *Proc. Natl. Acad. Sci. USA* **81**:5066–5070 (1984).
40. Safe, S., S. Bandiera, T. Sawyer, L. Robertson, A. Parkinson, P. E. Thomas,

D. Ryan, L. M. Reik, W. Levin, M. A. Denomme, and T. Fujita. PCBs: structure-function relationships and mechanism of action. *Environ. Health Perspect.* **60**:47–56 (1985).
41. Fagan, J. B., J. V. Paskwka, S. C. Chalberg, E. Gozukara, F. P. Guengerich, and H. V. Gelboin. Noncoordinate regulation of the mRNAs encoding cytochromes P-450$_{BNF/MC-B}$ and P-450$_{ISF/BNF-G}$. *Arch. Biochem. Biophys.* **244**:261–272 (1986).
42. Kawajiri, K., O. Gotoh, Y. Tagashira, K. Sogawa, and Y. Fujii-Kuriyama. Titration of mRNAs for cytochrome P-450c and P-450d under drug inductive conditions in rat livers by their specific probes of cloned DNAs. *J. Biol. Chem.* **259**:10145–10149 (1984).
43. Hardwick, J. P., P. Linko, and J. A. Goldstein. Dose-response for induction of two cytochrome P-450 isozymes and their mRNAs by 3,4,5,3′,4′,5′-hexachlorobiphenyl indicating coordinate induction in rat liver. *Mol. Pharmacol.* **27**:676–682 (1985).
44. Gonzalez, F. J., R. H. Tukey, and D. W. Nebert. Structural gene products of the Ah locus. Transcriptional regulation of cytochrome P_1-450 and P_3-450 mRNA levels by 3-methylcholanthrene. *Mol. Pharmacol.* **26**:117–121 (1984).

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