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PRINCIPAL INVESTIGATOR: Thomas A. Gasiewicz, Ph.D.

CONTRACTING ORGANIZATION: University of Rochester
Rochester, New York 14642

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Rochester, New York 14642

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13. ABSTRACT *(Maximum 200 words)*

The Ah receptor (AhR), a gene regulatory protein, has been implicated to play a significant role in the development and/or progression of mammary tumors in humans and animals. We further hypothesize that it plays a role in the normal development of breast tissue. The purpose of this grant was to define the cell- and developmentally-related expression and activation of the AhR in mammary tissue, determine the ability of AhR agonists to affect the expression of the estrogen receptor (ER) in this tissue, and define the mechanism(s) by which these events occur. We have shown that the human ER structural gene contains specific DNA sequences that bind the activated human AhR under conditions in vitro. We have sought to establish an in vivo model to further investigate the mechanisms whereby the AhR may regulate the ER gene. In contrast to previous investigations by others, we found that the AhR agonist, TCDD, did not affect either ER levels or estrogen-induced responses in the weanling female rat. These data indicate that age and/or developmental period play a crucial role in the ability of TCDD, via the AhR, to modulate the ER and estrogen-mediated responses. We are also in the final stages of developing a transgenic mouse model that will be extremely useful to examine the role of the AhR in normal and abnormal breast tissue development.

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Thomas A. Fleming 11/24/97

PI - Signature Date

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A. INTRODUCTION

The estrogen receptor (ER) and the response of this receptor to estrogenic compounds influence not only the molecular events that account for the development and progression of breast cancer, but also the response of this disease to a variety of therapeutic measures. As such, it is important to determine what factors may regulate the expression and actions of this receptor in breast tissue. Previous results have shown that the activation of another transcription factor, the Ah receptor (AhR), influences the spontaneous generation of mammary tumors in laboratory animals, alters estrogen-induced responses, and affects the ER content of tissues. Certain xenobiotics, e.g. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and structurally-related compounds, have been shown to activate the AhR to a DNA-binding state and elicit a variety of AhR-mediated biochemical and biological responses. Although the exact normal function of the AhR and its endogenous ligand are not known, the biological responses which TCDD and the halogenated aromatic hydrocarbons elicit indicate this protein serves some normal role in the control of processes involved in tissue differentiation and proliferation.

We hypothesize that the AhR plays an important role in the normal development of breast tissue. Furthermore, the abnormal regulation of the activity of this transcription factor may play a critical role in breast tumor development. We believe that AhR agonists, such as TCDD, cause down-regulation of the ER *in vivo* by binding to regulatory regions of the ER structural gene and decreasing the rate of its transcription.

The objectives of this research are three-fold: **1)** to determine the cell- and developmentally-related expression and activation of the AhR in rat mammary tissue, **2)** to examine the ability of AhR agonists to affect the expression of the ER in mammary tissue; and **3)** to examine the mechanism by which the AhR down-regulates the expression of the ER structural gene.

B. BODY

B.1. Examine the mechanism by which the AhR down-regulates expression of the ER structural gene. We have shown previously that the human ER structural gene contains specific DNA sequences (DREs, dioxin-responsive elements) that bind activated mouse and human AhR under conditions *in vitro* (White & Gasiewicz, 1993). We proposed that the AhR may regulate ER expression through the direct modulation of gene transcription. To prove this, it is necessary to show that this

regulation occurs under whole cell and/or *in vivo* conditions. Therefore, the initial goal was to establish a model to further investigate the mechanism of this down-regulation.

In our initial experiments in collaboration with Dr. A. Notides and using antibodies directed against the ER, we found that TCDD did not cause a decreased expression of the ER protein in human breast MCF7 cells. However, others have reported an effect on ER protein *in vivo* (Astroff and Safe, 1988; Romkes and Safe, 1988; Astroff et al., 1990; Hruska and Olson, 1989). Furthermore, it is likely that the particular response may be a function of tissue-, cell-, and/or temporal-specific ratios of various transcription factors. Therefore, we sought to establish a rat model to further investigate the mechanism for the antiestrogenic effects of TCDD.

TABLE 1

Effect of Estradiol and TCDD on Uterine, Thymic, and Hepatic Weights in Weanling Female Rats [a]

Treatment groups	Uterus weight (mg/kg BW) [b]	Thymus weight (mg/kg BW)	Liver weight (% BW)
Control (n=19)	0.73 ± 0.19	3.56 ± 0.29	4.58 ± 0.34
Estradiol (E_2) (n=20)	1.60 ± 0.23 [c]	3.59 ± 0.79	4.61 ± 0.16
E_2 + 20 µg/kg TCDD (n= 12)	1.68 ± 0.22 [c]	2.42 ± 0.29 [d]	5.44 ± 0.55 [d]
E_2 + 40 µg/kg TCDD (n=19)	1.61 ± 0.24 [c]	1.95 ± 0.26 [d]	5.49 ± 0.58 [d]
E_2 + 80 µg/kg TCDD (n=20)	1.74 ± 0.27 [c]	2.21 ± 0.50 [d]	5.49 ± 0.58 [d]

[a] Rats were treated sc with olive oil or TCDD on day 19, followed by treatment on days 21 and 22 with olive oil or 10 µg/kg/day E_2, as shown. All rats were euthanized on day 23.

[b] Results are the mean ± SD of a total of n animals from three separate experiments.

[c] Significantly different from control, $p < 0.001$.

[d] Significantly different from corresponding controls and E_2 treated, $p \leq 0.001$.

The effects of estradiol (E2) alone or TCDD plus E2 on several E2-dependent parameters were evaluated in weanling female Sprague-Dawley rats. E2 treatment (10 µg/kg/day at post-natal days (PND) 22 and 23) caused significant increases in relative uterine weight (Table 1) and keratinization of the vaginal epithelium (Fig. 1). E2 treatment significantly reduced uterine ER protein levels and serum FSH levels (Table 2), with a trend toward reduction of ER mRNA levels (Fig. 2). None of these parameters were affected by pretreatment with TCDD at PND 19. Uterine

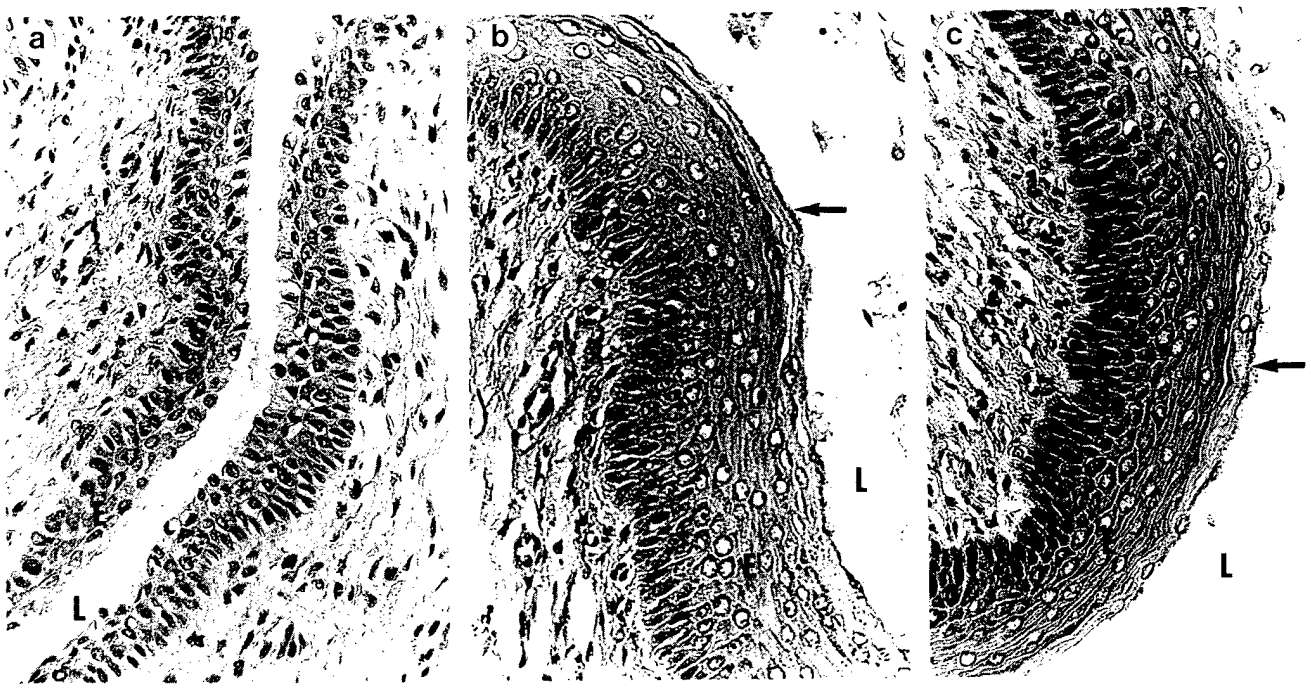

Fig. 1. Photomicrographs of vaginal epithelia. Rats were injected sc with olive oil or 10 µg/kg/day E2 on days 21 and 22, or E_2 (days 21 and 22) plus a pretreatment of TCDD (20, 40, or 80 µg/kg) on day 19. All animals were euthanized on day 23. Vaginal epithelium (E) from **(a)** a control rat, showing a transitional morphology; **(b)** an E_2-treated rat, showing a stratified, squamous morphology and producing abundant amounts of keratin (arrow); or **(c)** a rat treated with E_2 plus 80 µg/kg TCDD, also showing a keratinizing epithelium. Results were confirmed in two separate experiments, or a total of 8 animals per group (4 animals in the 20 µg/kg TCDD dose group). L, vaginal lumen; 310 x magnification.

TABLE 2
Effects of E2 and TCDD on Uterine Estrogen Receptor and Progesterone Receptor Content and Serum FSH Levels [a]

Treatments	Estrogen receptor (fmol/mg protein)[b]	Progesterone receptor (fmol/mg protein)[c]	Serum FSH (ng/ml)[d]
Control	632.8 ± 164.7 (7) [e]	191.2 ± 24.8 (7)	6.8 ± 2.4 (17)
E_2	404.9 ± 63.8 (8) [f]	188.3 ± 86.7 (8)	4.1 ± 1.1 (19) [g]
E_2 + 20 TCDD	472.9 ± 117.3 (4)	183.6 ± 29.5 (4)	3.7 ± 1.0 (10) [g]
E_2 + 40 TCDD	418.3 ± 122.2 (7) [h]	193.9 ± 94.0 (7)	5.1 ± 1.8 (18) [i]
E_2 + 80 TCDD	459.9 ± 99.1 (8) [h]	208.3 ± 83.7 (8)	4.3 ± 1.3 (15) [g]

[a] Rats were treated as in Table 1. Dose levels of TCDD are in µg/kg.
[b] Estrogen receptor levels were analyzed by EIA.
[c] Progesterone receptor levels were analyzed using a ligand binding assay.
[d] FSH serum levels were analyzed by RIA.
[e] All values are expressed as means ± SD for (n) number of animals.
[f] Significantly different from control, $p = 0.003$.
[g] Significantly different from control, $p \leq 0.002$.
[h] Significantly different from control, $p < 0.03$.
[i] Significantly different from control, $p = 0.03$; significantly different from E_2 treated, $p = 0.043$.

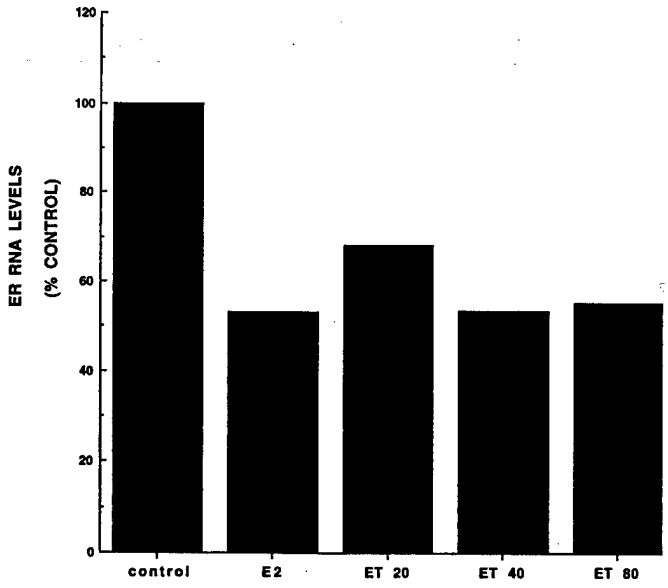

Fig. 2. Estrogen receptor mRNA levels. Animals were treated as in Fig. 1. Pairs of uteri within a treatment group were pooled, and total RNA was isolated, serially diluted onto a Nytran membrane, and analyzed using a slot blot hybridization procedure. For each RNA sample, values were initially plotted as PhosphorImager units vs amount of RNA loaded. Mean PhosphorImager values for 10 μg total RNA were calculated from the linear portions of the curves. Values are expressed as percentages of control and represent the mean from two RNA samples per treatment from one experiment. Treatments: E_2, estradiol alone; ET 20, E_2 + 20 μg/kg TCDD; ET 40, E_2 + 40 μg/kg TCDD; ET 80, E_2 + 80 μg/kg TCDD.

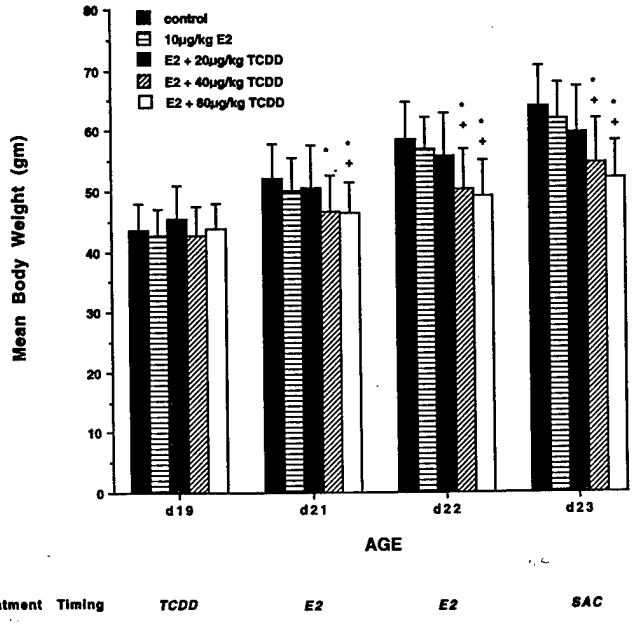

Fig. 3. Body weights of animals throughout the experiment. Animals were treated as in Fig. 1 and weighed on the day of each injection and at the time of euthanasia. Days of injection with either E_2 or TCDD are indicated. * Significant difference from control, $p < 0.01$; + significant difference from E_2 treatment, $p \leq 0.002$.

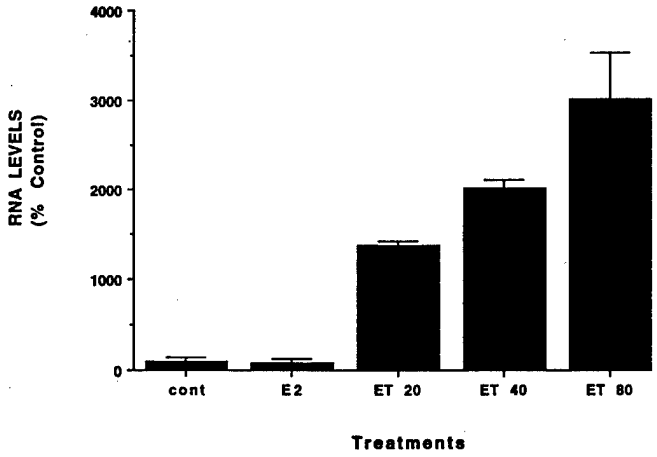

Fig. 4. CYP1A1 mRNA levels. Animals were treated as in Fig. 1. Total hepatic RNA was isolated from single liver samples, and slot blots were prepared. Mean PhosphorImager units were calculated as in Fig. 2. Values are expressed as percentages of control and represent the results of one experiment for a total of two samples per treatment group.

progesterone receptor levels were not affected by E2 or TCDD in this study (Table 2). In contrast, TCDD significantly decreased body weight by PND 21 (Fig. 3), significantly decreased relative thymic weights (Table 1), and signficantly increased hepatic weights (Table 1). In addition, TCDD dramatically induced hepatic cytochrome P450 1A1 mRNA levels (Fig. 4), indicating that TCDD was properly delivered and could mediate other well-documented AhR-dependent events. (The details of these results are contained in a recent publication (White et al., 1995) included in the Appendix.

Previous investigations by others have shown an effect of TCDD on E2-induced uterine weights and ER proteins levels. We interpret our data to indicate that age and/or developmental period play a crucial role in the ability of E2 to elicity biological effects, as well as the ability of TCDD via the AhR to interfere with these effects. Thus, while we, at least in this study, were unsuccessful in establishing a model to examine the effects of TCDD in the rat, the results are significant in pointing out the likely developmental specificity for the actions of the AhR.

As indicated above, the ability of TCDD, via the AhR, to regulate expression of the ER is likely cell- and temporal-specific. To further examine the specific mechanism whereby this occurs we again sought the use of an *in vitro* model that may not be dependent on factors *in vivo*. While we were in the process of performing these studies, another group of investigators established that treatment of a human ovarian carcinoma cell line, BG-1, with TCDD results in a dose-related depression of ER mRNA and protein (Clark, 1995). We were in the process of

obtaining these cells and developing the model to examine this mechanism more specifically. However, the individual (T. White) working on this project left this University. The particular aspect of this project has not been continued since an individual having expertise in this area was not available during the period of funding for this grant. We focused our attention on examining the developmentally-related expression and activation of the AhR in mammary tissue (see B.2 below).

B.2. Determine the cell- and developmentally-related expression and activation of the AhR in rat mammary tissue. Despite data indicating an affect of AhR agonists on the spontaneous generation of mammary tumors, the expression and cellular distribution of the AhR in this tissue has not been examined. These studies will provide important information suggesting a period in which the AhR may play a critical role in breast tissue development.

We have been working on the methodologies for immunohistochemistry of the AhR protein and *in situ* hybridization for the determination of AhR mRNA. Much difficulty has been enountered with the availability of appropriate anti-AhR antibodies and with the technique giving results that are both interpretable and consistent. We will be continuing the development of these procedures in the future, and will examine the developmental expression of the AhR in rat mammary tissue.

While the completion of the above studies will give some information on the tissue- and temporal-specific expression of the AhR it will give no information on the <u>functional activity</u> of this receptor in mammary tissues at various times of development and/or following exposure *in vivo*. It would be useful to couple this information obtained in Aim B.3 (see below) to determine the precise relationship with expression of the ER. The expression of *CYP1A1* and/or other inducible genes has most often been used to <u>indirectly</u> assess the ability of AhR agonists to activate this protein. However, it is clear that the induction of these, especially *CYP1A1*, may have little to do with the toxic responses observed or alteration of other genes, are tissue-specific, and may not concomitantly occur in all responsive cells and/or tissues. Furthermore, it is often technically difficult to assess these in a cell-specific manner and/or in developing tissues. Nuclear localization of the AhR complex has been used as a measure of AhR activation (Whitlock and Galeazzi, 1984; Gasiewicz et al., 1991). However, we know too little about the regulation of its subcellular distribution to assume that this localization is always indicative of a transcriptionally active AhR form. Finally, while immunohistochemistry and *in situ* hybridization can and have provided useful information about the tissue-specific presence of many proteins, the cross-reactivity of antibodies and mRNA probes with non-specific sites is often difficult to rule out. (In fact, we are having problems with just these issues.) Furthermore, expression does not always indicate transcriptional activity. Clearly, it is important to determine, regardless of expression, whether the AhR is transcriptionally active in a cell- and temporal-

specific manner. This information would help to define cellular targets and periods of sensitivity for the action of AhR agonists. To address these issues, we have begun to take an approach successfully used by others to study the regulation of other genes and receptors (Colbert *et al.*, 1993; Balkan *et al.*, 1992; Smeyne *et al.*, 1992), that will allow the determination of the tissue/cell and time of AhR activation to a transcriptionally active complex in breast tissue.

The goal of these studies is to develop a transgenic mouse model that will allow the determination of the precise time and tissue/cell-specific location for the presence of transcriptionally active AhR complex in developing and adult breast tisssue from TCDD-treated and untreated animals. The approach relies on **1)** the optimization of a reporter gene construct responsive to TCDD via the AhR complex, **2)** incorporation of this transgene (TG) into the mouse genome, **3)** the ability of the activated-AhR complex to bind to responsive elements present in the reporter construct *in vivo*, **4)** transcriptional enhancement of the particular TG, and **5)** sensitive detection of the reporter gene translation product (usually an enzyme activity) within tissues. Up to this time we have devoted much effort to 1) the characterization and optimization of a reporter gene system, 2) the creation of the transgenic mice carrying the reporter gene, and 3) characterization of the genotype and phenotype of the TG founder mice.

B.2.a. Characterization and optimization of a reporter gene system. The heterodimer AhR-Arnt complex has been found to specifically recognize and bind to *cis*-acting elements, dioxin-response elements (DREs), present in the 5′ regulatory regions of responsive genes to modulate rates of transcription. The core sequence for the DRE has been identified and is highly conserved in a number of mammalian species. Thus, a specific way to measure the level of activated AhR is through the use of a functional assay involving DREs linked to reporter genes. This approach has been used by a number of investigators to study AhR-mediated gene regulation in cultured cells. However, it is important for us to optimize the construct so that 1) the background level of the reporter gene activity is low, 2) the induced signal-to-background ratio is high, 3) the construct is responsive only to the activated AhR-Arnt complex, and 4) the reporter gene is easily detectable with high sensitivity. To test and optimize the construct in cultured cells we used the *luciferase* gene because of its proven utility as well as ease and sensitivity of detection. For *cis*-acting enhancer elements we chose to use a 32-bp fragment (Fig. 5 below) containing the DRE$_D$ as designated by Lusska *et al.* (1993). Notably, the 32-bp fragment is identical to that contained in the upstream region of the *CYP1A1* gene. Thus, it is a *bona fide* AhR-Arnt binding site and contains both the "core" recognition sequence + flanking sequences found in the mouse. The latter sequences also have been found to have a significant role in determining relative affinity of the AhR-Arnt complex and activity (Swanson *et al.*, 1995; Lusska *et al.*, 1993). Among the other DRE-containing sequences upstream of the *CYP1A1* gene that have been identified and tested, the DRE$_D$ has been indicated to be the most

active (Lusska *et al.*, 1993). Finally, a minimal promoter 42-bp fragment (Fig. 5) consisting of a TATA box from the chicken *ovalbumin* (*ova*) promoter (Bagchi *et al.*, 1990) was used. We purposefully avoided more complex promoters that may contain multiple elements of regulation, i.e. binding sites for other transcription factors.

5′-GATCTGAGCTCGGAGTTGCGTGAGAAGAGCCG-3′
32-b enhancer fragment containing the DRE$_D$ (underlined)

5′-AATTCAGATCTGAGGTCCACTTCGCTATATATTCCCCGAGCT-3′
42-b fragment of *ova* promoter containing the TATA box (underlined)

Fig. 5: Sequences for enhancer and promoter elements used in p2Dluc

The two synthetic DNA sequences (DRE$_D$ and lovTATA) were compared against all known sequences which are transcription factor sites by using the VAX/GCG command "findpatterns" after accessing the database "TFsites.dat", and using the "text searches" from NCBI's website in GenBank or Swiss-Prot. Using these searches, the DRE and TATA-box interactions were confirmed, but there were no other known complete transcriptional sites evident within these sequences.

The initial minimal promoter constructs to be tested were made by ligating two or six 32-bp DRE$_D$s to form multimers. Protruding ends were filled in with *Taq* polymerase before ligation into pCRII vector. Inserts that contained the DRE$_D$s were digested with *Eco*RI and cloned into plovTATA (Bagchi *et al.*, 1990) which contains a TATA box from the *ova* promoter. PCR primers were synthesized that flanked the *Eco*RI site and TATA box and contained new restrictions sites for *Xho*I and *Hind*III. The resulting PCR product was digested and cloned into pGL2-Basic Vector, creating pTATAluc, p2Dluc (Fig. 6), and p6Dluc with 0, 2 and 6 DRE$_D$s, respectively. The proper clones were verified by restriction digest mapping and sequencing. These constructs were transiently transfected into murine Hepa 1c1c7 (Hepa) cells. The cells were treated with TCDD (18h) to determine *luciferase* inducibility. The Luciferase Assay System and a luminometer were used to determine luciferase activity. Using these transfected cells, we determined that 1) the minimal ova promoter imparts low basal levels of luciferase activity and is not inducible by 2 nM TCDD (Fig. 7), 2) a construct containing 2 DREs and the ova promoter flanking the *luciferase* gene was optimal and imparted a 25- to 80-fold induction with 2nM TCDD (Fig. 7) (Note: Although the absolute level of induction was greater using the construct containing 6 DRE$_D$s, the background level of induction was also significantly higher, and thus the relative fold induction over the control was less (Fig. 7)), and 3) the construct containing 2 DREs was sensitive to 5 pM TCDD (Fig.8). Using a similar construct, a *thymidine kinase* (*TK*) promoter was also tested (instead of the *ova* promoter). Treatment with 2nM TCDD induced the *TK*- and *ova* promoter-containing constructs by 5- and 80-fold, respectively (not shown). (In subsequent work, the p2Dluc construct (2 DRE$_D$s, *ova* promoter, and

luciferase gene) transiently transfected into Hepa cells was successfully used to examine the AhR antagonist activity of a number of flavones and ellipticines (see Gasiewicz *et al.*, 1996; also in Appendix). These compounds will be useful to examine the activity of TCDD to modulate genes in breast tissue).

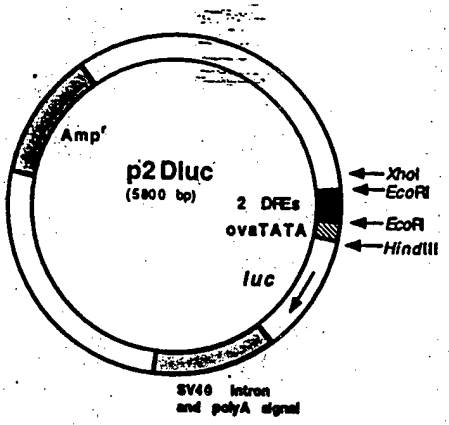

Fig. 6: Map of p2Dluc plasmid

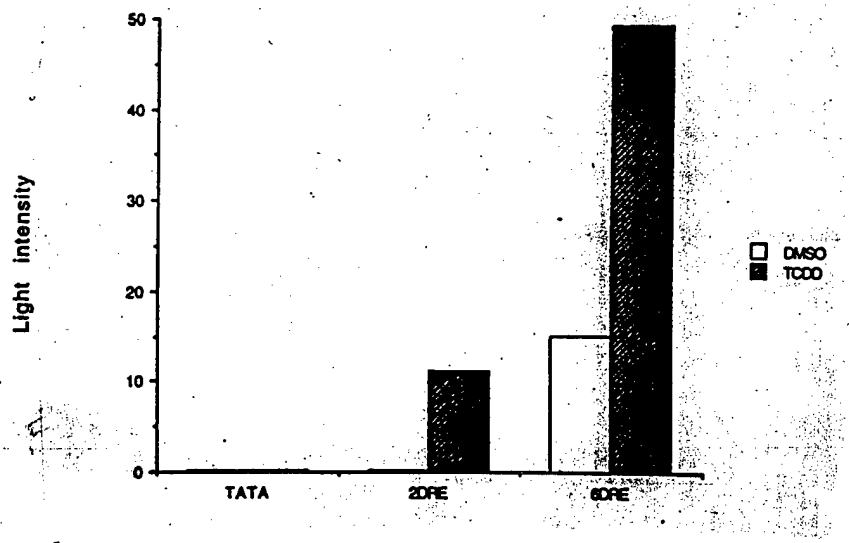

Fig. 7: Background expression of luciferase and response to 2 nM TCDD following transient transfection of constructs containing 0 (TATA), 2, and 6 DREs into Hepa cells. The ratios of results for TCDD/DMSO treatments were 0.6, 46, and 3.3 for 0, 2, and 6 DREs, respectively.

We have subsequently developed stable transfectants using the p2Dluc construct and Hepa cells. Analysis of these stable transfectants indicates 1) a very low background *luciferase* expression (Fig. 9), 2) high inducibility by TCDD (150- to 230-fold) over background expression at 1 nM TCDD (Fig. 9), and 3) high sensitivity to TCDD inducibility (significant induction was observed at 1.0 pM (Fig. 9)). These and the above data indicate that the construct meets our criteria for low background

expression, high sensitivity, and selectivity for the active AhR-Arnt complex.

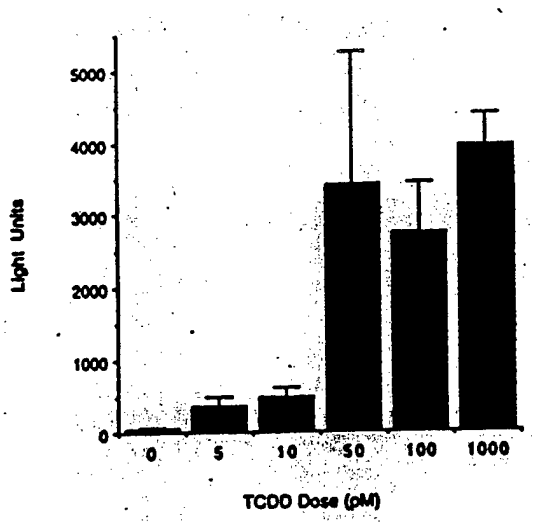

Fig. 8. Luciferase induction following transient lipofection of 2Dluc into Hepa cells and treatment (18) with DMSO or TCDD. S.D. are shown.

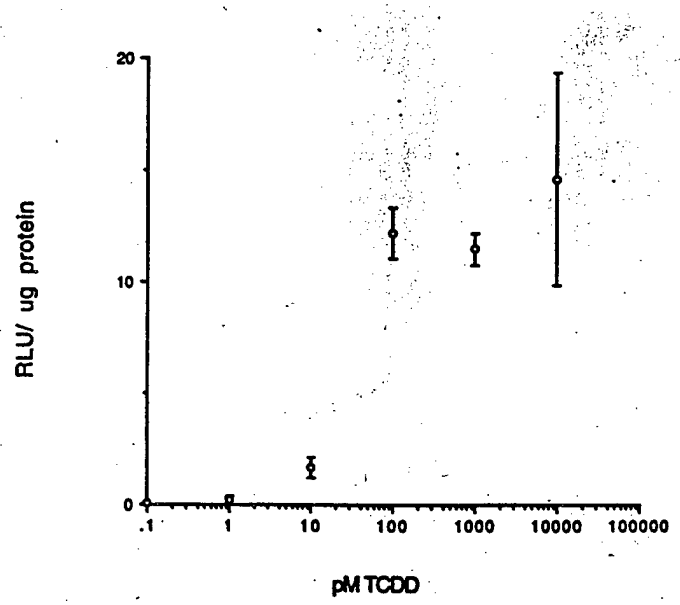

Fig. 9. Luciferase induction following stable transfection of 2Dluc into Hepa cells and treatment (18 h) with various concentrations of TCDD. S.D. are shown. 1.15- and 3.3-fold induction were observed at 0.1 and 1.0 pM, respectively.

While the assays for luciferase activity proved useful following transfection of the construct into isolated cells in culture, luciferase activity cannot readily be detected *in situ*. In similar models, *lacZ*, encoding bacterial β-galactosidase, has most often been used as the reporter transgene to determine tissue- and cell-specific

activity of particular transcription factors. Since the DRE-*ova* TATA promoter appeared to be sensitive, specific and efficient for driving AhR-mediated induction of the *luciferase* gene, we cloned the same promoter upstream of the *lacZ* gene to make our transgene construct. The source of the lacZ-SV40 intron and polyA sequence was the plasmid RARE-tk-Bgal (a gift from M. Colbert), which was successfully used to make retinoic acid responsive transgenic mice (Balkan *et al.*, 1992). Briefly, the 2.9 Kb *Hind*III-*Not*I fragment of pGEM 9z was first ligated to the 4 Kb *Hind*III-*Not*I fragment of RARE-tk-Bgal to remove the RAREs (retinoic acid response elements). The tk (thymidine kinase) promoter was removed by *Pst*I/*Xba*I digestion, leaving the *lacZ* vector into which the *Hind*III-*Xho*I promoter fragment from p2Dluc was blunt-end cloned, followed by the filling in of all protruding ends with the Klenow polymerase. The resulting plasmid, p2Dlac was confirmed by sequencing and restriction mapping. This was transiently transfected into Hepa cells. β-galactosidase was determined in cell lysates using *o*-nitrophenyl β-D-galactopyranoside (ONPG) as the substrate and *in situ* using X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactoside) as the substrate (not shown). Similar to the construct containing the *luciferase* gene, we found the 2DRE-*ova* promoter-*lacZ* gene construct to be exceptionally responsive to TCDD treatment (not shown). There was an approximate 80-fold induction at 2 nM TCDD and the limit of detectabililty was near 5 pM TCDD.

In summary, we have shown that the construct containing 2 DREDs, the *ova* promoter and the template DNA consisting of either the *luciferase* or *lacZ* structural genes or a G-free cassette have very low background expression in mouse hepatoma cells and under cell free conditions. In addition, this construct specifically responds to the agonist-occupied AhR-Arnt complex with a high level of sensitivity and inducibility.

B.2.b. The creation of transgenic mice carrying the DRE-*ova* promoter-*lacZ* contruct. The plasmid containing the DRE-*ova* promoter-*lacZ* construct was digested with *Sal*I and *Not*I. The 4 Kb fragment was gel purified, isolated, and further column purified and ethanol-precipitated. The DNA was shipped to DNX, Inc. (Princeton, NJ) for the microinjection into pronuclei of recently fertilized mouse (C57Bl/6 x SJL hybrid) ova and the creation of transgenic mice. Thirty-three potential founders were supplied to us by DNX, Inc. (see Table 3). Nine of these were found to be TG^+ as determined by a PCR-based assay that we developed using primers as previously described (Buzy et al., 1995) to amplify a 525 bp fragment of *lacZ*. Eight of the 9 were found to be TG_+ as determined by Southern blot analysis (not shown). Copy number ranged from 1 to >100 (Table 3). These 8 were bred with C57Bl/6 mice at the University of Rochester Environmental Health Sciences Center Breeding Facility to yield hemizygous litters of various sizes (Table 3). Some of the mice were rebred due to small litter sizes (#s 7, 16, and 22), or low frequency of transgene expression (# 9). The other potential founders are either presently pregnant or have been rebred to determine the consistency of transgene expression

in the litters. Results from 6 of the 8 breedings, i.e. for mice #s 1, 7, 18, 21,22, and 27, indicate an approximate 50% TG expression in the offspring. This suggests integration of the TG into a single site. The frequency of expression in the litter resulting from the litter of #9 was low. This might result from the incorporation of the TG into only a subset of the germ cells. Finally, the relatively high TG+ expression the litter from the breeding of #16 suggests that possibility that integration of the TG had occurred at more than one site. Additional breedings of this founder are in process to confirm an increased frequency of TG expression. In addition, we are proceeding with an analysis of these mice to determine the expression of the TG in tissues, including mammary tissue, in the absence and presence of TCDD.

TABLE 3: Screening and breeding of mice obtained from DNX, Inc.

MOUSE #	PCR SIGNAL	COPY #	SEX	# PUPS/litter	Transgene Frequency (%TG+)
1	++	>100	M	7	43
2-4	-	0	M	-	
5-6	-		M	-	
7	+	<10	M	1*	100
				11	64
8	-		M	-	
9	+	<10	M	6*	0
				10	20
10-14	-		M	-	
15	+	0	M	-	
16	++	>10	M	5*	60
				9	78
17	-		M	-	
18	+	1	M	6	50
19-20	-		F	-	
21	+	1	F	8	38
22	++	<10	F	2*	50
				12	50
23-26	-		F	-	
27	++	10	F	7	43
28-33	-		F	-	

No numbers under "copy #" indicates no analysis was performed due to the lack of PCR signal. A "0" under "copy #" indicates that an analysis was performed but no signal was observed.

* These were rebred.

B.3. Examine the ability of AhR agonists to affect the expression of the ER in mammary tissue. These studies will complement the previous objective by suggesting a relationship between AhR expression in this tissue and the regulation of ER protein and mRNA expression as affected by AhR agonists. These studies are very dependent on those in the previous objective. Furthermore, the studies

presently underway as outlined in B.1. will also assist in designing the protocols for these. As such, we have not yet begun these studies.

C. CONCLUSIONS

The results to date are important in emphasizing the tissue- and age-dependent nature of the effects of TCDD on E2-dependent responses. Thus, these data indicate that if the AhR does have some role in regulating the ER and ER-dependent responses, it is likely to be developmental specific. This in fact could have important implications for the normal and abnormal development of breast tissue.

We are in the final stages of developing a transgenic mouse model that we believe will be extremely useful to examine the role of the Ah receptor in normal and abnormal breast tissue development.

Future work will specifically focus on breast tissue for the presence and actions of the AhR specifically as it may regulate the expression and actions of the ER (and other genes affecting the actions of E2 in this tissue).

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E. Appendix.

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Weanling Female Sprague–Dawley Rats Are Not Sensitive to the Antiestrogenic Effects of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)

T. E. K. WHITE, G. RUCCI, Z. LIU, AND T. A. GASIEWICZ[1]

Department of Environmental Medicine, University of Rochester Medical Center, 575 Elmwood Avenue, Rochester, New York 14642

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Weanling Female Sprague–Dawley Rats Are Not Sensitive to the Antiestrogenic Effects of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD). WHITE, T. E. K., RUCCI, G., LIU, Z., AND GASIEWICZ, T. A. (1995). *Toxicol. Appl. Pharmacol.* 133, 313–320.

Investigators have shown that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) can inhibit certain estrogenic events *in vivo* and *in vitro*. To further investigate this phenomenon, the effects of estradiol (E_2) alone or TCDD plus estradiol on several estrogen-dependent parameters were evaluated in weanling female Sprague–Dawley rats. E_2 (10 μg/kg/day, Postnatal Days (PND) 21 and 22) caused significant increases in relative uterine weight and keratinization of the vaginal epithelium (PND 23). E_2 significantly reduced uterine estrogen receptor (ER) protein levels and serum FSH levels, with a trend toward reduction of ER mRNA levels. None of these parameters were affected by pretreatment with 20, 40, or 80 μg/kg TCDD (PND 19). Uterine progesterone receptor levels were not affected by E_2 or TCDD in the present study. In contrast, TCDD significantly decreased body weight (40 or 80 μg/kg) by PND 21, significantly decreased relative thymic weights, and significantly increased relative hepatic weights (20, 40, and 80 μg/kg, by PND 23). In addition, TCDD dramatically induced CYPIA1 hepatic mRNA levels, indicating that TCDD was properly delivered and could mediate other well-documented Ah receptor-dependent events. Thus, weanling female Sprague–Dawley rats are not sensitive to the antiestrogenic effects of TCDD at doses which cause overt toxicity. The results provide evidence that the previously reported antiestrogenic effects of TCDD are probably species, strain, and age dependent.

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the most toxic member of the halogenated aromatic hydrocarbons. The pattern and severity of TCDD toxicity may vary with species, sex, and age, depending on the endpoint examined. In experimental animals, TCDD commonly causes a wasting syndrome resulting in delayed death, immunosuppression and thymic atrophy, liver damage, chloracne, and alterations in growth and differentiation of epithelial tissues (Poland and Knutson, 1982). At the molecular level, TCDD activates the aryl hydrocarbon receptor (AhR), which is a ligand-activated transcription factor. Upon activation, the AhR acquires DNA binding specificity and enhances the activity of a number of genes, most notably the CYPIA1 (cytochrome P-450IA1) gene (Poland and Knutson, 1982; Gasiewicz, 1991).

Recently, considerable attention has focused on TCDD's reproductive and antiestrogenic effects. TCDD has been shown to interfere with the maintenance of pregnancy, as well as fetal growth, development, and viability in rats (Giavini *et al.*, 1983; Murray *et al.*, 1979) and mice (Umbreit *et al.*, 1987). In addition, mice exposed to TCDD-contaminated soils exhibited a cessation of estrous cycling (Umbreit *et al.*, 1987). Female rhesus monkeys failed to conceive after TCDD exposure, and significant alterations in hormone levels were observed (Barsotti *et al.*, 1979). It has been suggested that TCDD affects reproduction by causing an alteration in the action of estrogens (Umbreit and Gallo, 1988), and indeed, TCDD has been reported to show some antiestrogenic actions. For example, TCDD inhibited estrogen-induced increases in uterine wet weights and endometrial development (Gallo *et al.*, 1986) and decreased hepatic and uterine estrogen receptor (ER) levels in immature mice (Lin *et al.*, 1991; DeVito *et al.*, 1992a,b). Similarly, TCDD reduced basal and estradiol (E_2)-stimulated ER levels in rats (Romkes *et al.*, 1987; Astroff and Safe, 1988; Romkes and Safe, 1988) and interfered with E_2-induced increases in uterine progesterone receptor levels and epidermal growth factor receptor activity and expression (Astroff and Safe, 1988; Romkes and Safe, 1988; Astroff *et al.*, 1990). Hruska and Olson (1989) demonstrated that the effects on ER levels were species and tissue specific, with hamsters, guinea pigs, and rats showing different patterns of TCDD-induced changes in hepatic and uterine ER.

A mechanism for the antiestrogenic effects of TCDD *in vivo* has not been elucidated. *In vitro,* TCDD-induced inhibition of estrogen-dependent growth and extracellular tissue plasminogen activator secretion by MCF-7 (human breast carcinoma) cells has been correlated with alterations in E_2 metabolism (Gierthy *et al.*, 1987; Spink *et al.*, 1990). Alterations in serum estrogen levels after TCDD treatment

[1] To whom correspondence should be addressed at Department of Environmental Medicine, Box EHSC, University of Rochester Medical Center, 575 Elmwood Avenue, Rochester, NY 14642. Fax: (716) 256-2591.

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have not been documented *in vivo* (Shiverick and Muther, 1982, 1983), but this does not preclude changes in intracellular levels of estrogen and/or its metabolites. Although decreases have been noted in nuclear ER levels in MCF-7 cells after pretreatment with TCDD (Harris *et al.*, 1990; Wang *et al.*, 1993), these effects represent changes in initial nuclear uptake of liganded receptor and cannot necessarily be extrapolated to the decrease in total ER levels observed 48 hr after E_2 exposure *in vivo* (Romkes *et al.*, 1987; Romkes and Safe, 1988).

The initial goal of the present study was to establish a rat model to further investigate a mechanism for the antiestrogenic effects of TCDD. However, under the present experimental conditions, although E_2 elicited estrogenic effects, these could not be prevented by TCDD, even at doses which were well above the reported LD50 for this strain (Seefeld *et al.*, 1984) and which produced overt toxic changes. These results have important implications for considering the mechanisms by which TCDD may produce antiestrogenic effects in other biological systems.

MATERIALS AND METHODS

Chemicals. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (>98% pure) was purchased from Cambridge Isotopes (Cambridge, MA). [17α-methyl-^{3}H]-promegestone ([^{3}H]R5020; 84 Ci/mmol) and unlabeled R5020 were purchased from DuPont NEN (Boston, MA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Treatment of animals. Postnatal Day (PND) 14 female Sprague–Dawley rats were obtained from Charles River (Wilmington, MA), in groups of 10, with a lactating mother. Animals were maintained on a 12-hr light/dark cycle, with food and water provided *ad libitum.* On PND 19, animals were weaned and randomly separated into five groups: control, 10 μg/kg E_2, or E_2 plus 20, 40, or 80 μg/kg TCDD. Three separate experiments were performed, for a total of 12–20 animals per treatment group. Rats received a single sc injection of TCDD or olive oil vehicle (2.5 ml/kg) on PND 19, followed by two consecutive sc doses of E_2 or olive oil on PNDs 21 and 22. On PND 23, animals were killed by CO_2 overdose, blood was drawn by heart puncture, and uteri, livers, and thymus glands were excised and weighed. Body weights were recorded on every treatment day and at the time of termination. Uteri designated for ER or progesterone receptor (PR) analysis were snap frozen individually in 2 vol TEEDG (40 m*M* Tris, pH 7.4, at 4°C, 1 m*M* EDTA, 1 m*M* EGTA, 1 m*M* dithiothreitol, 10% glycerol, 0.5 μg/ml leupeptin, 1.5 μg/ml chymostatin, 1.5 μg/ml pepstatin, 50 m*M* NaF) and stored at −80°C until used. Uteri designated for RNA analysis were paired, homogenized in GI (4 *M* guanidine isothiocyanate, 25 m*M* sodium citrate, 0.5% sarkosyl, 0.1 *M* β-mercaptoethanol) (Chomczynski and Sacchi, 1987), snap frozen in liquid N_2, and stored at −80°C until used. One gram of each liver was homogenized in GI solution, snap frozen, and stored at −80°C.

Uterine whole-cell extracts. Frozen uterine samples were partially thawed, and phenylmethylsulfonylflouride (PMSF) was added to a concentration of 0.2 m*M*. Samples were homogenized, and the KCl concentration was raised to 0.42 *M*. Homogenates were kept on ice for 1 hr (with vortexing every 15 min) and then centrifuged for 1 hr at 100,000*g*. Supernatants were collected, aliquoted, and stored at −80°C until used.

ER and PR determinations. Total ER content in uterine whole-cell extracts was determined using an ER–enzyme immunoassay (ER–EIA) diagnostic kit (Abbott Laboratories, Chicago, IL), according to manufacturer's instructions. PR content in uterine whole-cell extracts was measured using a ligand binding assay (Horwitz and McGuire, 1978) as follows. Whole-cell extracts were diluted to 1 mg/ml protein in TEEKD (40 m*M* Tris, pH 7.4, at 4°C, 1 m*M* EDTA, 1 m*M* EGTA, 100 m*M* KCl, 1 m*M* dithiothreitol) and incubated overnight at 4°C with 10 n*M* [^{3}H]R5020 plus (nonspecific binding) or minus (total binding) 100-fold excess of unlabeled R5020 (added 15 min prior to the addition of the [^{3}H]R5020). Aliquots (200 μl) of ligand-containing extracts were incubated (0°C), in triplicate, with 250 μl of a 50% slurry of hydroxylapatite (HAP) in TEEKD, and the mixtures were vortexed every 5 min for 30 min. The HAP was pelleted by centrifugation at 800*g*, and the HAP pellets washed six times with TEEKD. The pellets were transferred to scintillation vials with two 1-ml aliquots of absolute ethanol and counted in a β-scintillation counter. Specifically bound ligand was determined by subtracting nonspecific counts from total counts, and the PR concentration calculated based on binding of one molecule R5020 to one molecule of PR. Results were expressed as fmol PR/mg protein.

Isolation of total uterine and hepatic RNA. Total RNA was isolated using a modification of the method of Chomczynski and Sacchi (1987). Briefly, uteri were homogenized in 30 ml GI per gram tissue, extracted twice with equal volumes of phenol and chloroform, and ethanol precipitated. Following resuspension in 500 μl GI, a third phenol/chloroform extraction was performed, followed by the final ethanol precipitation. RNA was quantitated by absorbance at 260 nm. For isolation of total liver RNA, samples were homogenized in 10 ml GI per gram, and a single phenol/chloroform extraction was performed, followed by ethanol precipitation. Precipitates were resuspended in 500 μl GI and ethanol precipitated. RNA samples were stored in ethanol at −20°C until the day of analysis, at which time they were centrifuged, washed with 80% ethanol, and resuspended in diethylene pyrocarbonate (DEPC, Sigma)-treated water. RNA concentrations were estimated by measuring absorbance at 260 nm.

Analysis of ER mRNA. Twenty micrograms of total uterine RNA was separated on a 1% agarose gel (6% formaldehyde, 10 m*M* sodium phosphate buffer, pH 7.4) and capillary-transferred overnight to a Nytran (Schleicher and Schuell, Keene, NH) nylon membrane. RNA was uv-crosslinked to the membrane using a UV Stratalinker 1800 (Stratagene, La Jolla, CA). The membrane was prehybridized at 65°C in hybridization buffer (1× SSPE, 1.5× Denhardt's, 10% dextran sulfate, 2% SDS, 100 μg/ml fragmented and denatured salmon sperm DNA) for 1–2 hr, fresh hybridization buffer was added containing 1–2 × 10^6 cpm/ml [^{32}P]ER cDNA probe, and hybridization was carried out overnight at 65°C. The probe employed was a 150-bp portion of a human ER cDNA (Green *et al.*, 1986) (generous gift of Dr. Pierre Chambon, Strasbourg, France), which was random-primer labeled with [^{32}P]dCTP (6000 Ci/mmol, NEN) using the Amersham Multiprime Labeling System (Amersham, Arlington, IL). For slot blot analysis, RNA samples were denatured in 7.4% formaldehyde and 6× SSC for 15 min at 65°C, serially diluted, and loaded onto a Nytran membrane using a Hoefer PR 648 slot blot filtration manifold (Hoefer Scientific, San Francisco, CA). Slot blots were hybridized with the 150-bp ER probe as above and quantitated using a Model PSI PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Analysis of CYPIA1 mRNA levels. Total hepatic RNA was isolated, and 20 μg RNA blotted onto a Nytran membrane using a slot blot aparatus, as described above. A ^{32}P-labeled riboprobe, corresponding to a region from +1965 to +2294 of the mouse CYPIA1 gene (>90% sequence homology to the rat), was synthesized using a T7 Maxiscript kit (Ambion). The blot was prehybridized and hybridized as above, in a formamide-containing hybridization buffer (50% deionized formamide, 3× Denhardt's solution, 3× SSPE, 0.2% SDS, 100 μg/ml denatured salmon sperm DNA).

Serum FSH levels. After blood was drawn by heart puncture, blood samples were spun at 1000*g* for 20 min, and serum was withdrawn and stored at −80°C. Serum FSH levels were measured in the laboratories of Dr. Patricia Rodier and Dr. Richard K. Miller using a RIA kit for rat FSH (rFSH) obtained from the NIDDK containing the following materials: anti-rat FSH-S-11 antisera (AFP-C0972881), rat FSH-I-8, IOD (AFP-11454B), rat FSH-RP-2 (AFP-4621B).

TABLE 1
Effect of Estradiol and TCDD on Uterine, Thymic, and Hepatic Weights in Weanling Female Rats[a]

Treatment groups	Uterus weight (mg/kg body wt)[b]	Thymus weight (mg/kg body wt)	Liver weight (% body wt)
Control ($n = 19$)	0.73 ± 0.19	3.56 ± 0.29	4.58 ± 0.34
Estradiol (E_2) ($n = 20$)	1.60 ± 0.23[c]	3.59 ± 0.79	4.61 ± 0.16
E_2 + 20 μg/kg TCDD ($n = 12$)	1.68 ± 0.22[c]	2.42 ± 0.29[d]	5.44 ± 0.55[d]
E_2 + 40 μg/kg TCDD ($n = 19$)	1.61 ± 0.24[c]	1.95 ± 0.26[d]	5.49 ± 0.58[d]
E_2 + 80 μg/kg TCDD ($n = 20$)	1.74 ± 0.27[c]	2.21 ± 0.50[d]	5.49 ± 0.58[d]

[a] Rats were treated sc with olive oil or TCDD on Day 19, followed by treatment on Days 21 and 22 with olive oil or 10 μg/kg/day E_2, as shown. All rats were euthanized on Day 23.
[b] Results are the mean ± standard deviation of a total of n animals from three separate experiments.
[c] Significantly different from control, $p < 0.001$.
[d] Significantly different from corresponding controls and E_2 treated, $p \leq 0.001$.

RESULTS

Effect of TCDD on estrogenic parameters. The ability of TCDD to antagonize estrogen-dependent processes was investigated in weanling female Sprague–Dawley rats. Two consecutive sc injections of 10 μg/kg/day 17-β-estradiol (E_2) on PNDs 21 and 22 caused a dramatic increase in uterine wet weight, as a proportion of body weight, to 219% of control by PND 23 (Table 1). Pretreatment with 20, 40, or 80 μg/kg TCDD (PND 19) did not prevent this uterotrophic effect (Table 1). Within each experiment, TCDD also did not change absolute uterine weights relative to E_2 treatment alone (data not shown). Morphologically, control animals showed a transitional vaginal epithelium (Fig. 1a), whereas E_2-exposed animals showed a stratified squamous epithelium, which produced abundant amounts of keratin (Fig. 1b). All E_2/TCDD cotreated animals showed a stratified keratinizing vaginal epithelium, indicating that TCDD did not inhibit this estrogen-dependent process. Figure 1c shows an example of the effects of 80 μg/kg TCDD plus E_2 on vaginal epithelial morphology. Uterine growth (Hisaw, 1959) and vaginal keratinization (Bertalanffy and Lau, 1963) are two well-documented responses to E_2 in the rat, which were clearly not affected by TCDD in the present study.

The effects of E_2 or E_2 plus TCDD on uterine ER protein and mRNA levels were also investigated. Table 2 shows that E_2 treatment significantly decreased total immunodetectable ER protein. Cotreatment with TCDD did not cause any significant changes in uterine ER levels relative to E_2 exposure alone (Table 2). As expected, uterine ER levels in the 40 and 80 μg/kg TCDD groups were significantly lower than control levels; however, ER levels in the 20 μg/kg group were slightly elevated, such that they were not significantly different from controls. This trend was reflected in uterine ER mRNA levels, which appeared to be decreased after E_2 treatment, but which were unaffected by TCDD pretreatment (Fig. 2). The E_2-induced decrease in uterine ER levels is in contrast to the increase demonstrated by others (Romkes *et al.*, 1987; Romkes and Safe, 1988). However, it is in good agreement with Medlock and colleagues (1991), who showed that estradiol downregulates uterine ER in Sprague–Dawley rats implanted with estradiol-containing silastic capsules. The difference might be explained on the basis of time of euthanization relative to estradiol exposure, since the latter group also showed that after withdrawal from estrogen, uterine ER levels could return to, then increase above, ER levels in control animals (Medlock *et al.*, 1991).

FSH release by the pituitary is under negative regulation by E_2 in the adult (Fox and Laird, 1970), and the same should be true in the weanling (Ojeda *et al.*, 1986). In the present study in weanling animals, E_2 caused a significant decrease in serum FSH levels (Table 2). Cotreatment with TCDD did not generally alter FSH levels relative to the E_2 alone group (Table 2), except for a slight, but significant, rise at the 40 μg/kg dose level. Neither E_2 nor E_2 plus TCDD had any significant effects upon uterine progesterone receptor content under the present experimental conditions (Table 2). Research has shown that 4 to 9 days of continuous estrogen exposure may be necessary for maximal induction of PR *in vitro* (Horwitz and McGuire, 1978; Sumida *et al.*, 1988). Therefore, the inability of a 2-day estrogen exposure to change PR levels *in vivo* is not surprising.

Effects of TCDD on general toxicity endpoints. To ensure that the animals were being appropriately dosed with TCDD under the current experimental paradigm, several other general effects of TCDD were measured. Whereas E_2 had no effect upon body weight gain, TCDD significantly lowered growth rate in a dose-dependent manner (Fig. 3). This effect was significant for the 40 and 80 μg/kg TCDD groups by 2 days after TCDD exposure (PND 21) (Fig. 3). A decrease in growth rate in weanling animals is analogous to the decrease in body weights observed in older animals as part of the TCDD-induced wasting/delayed death syn-

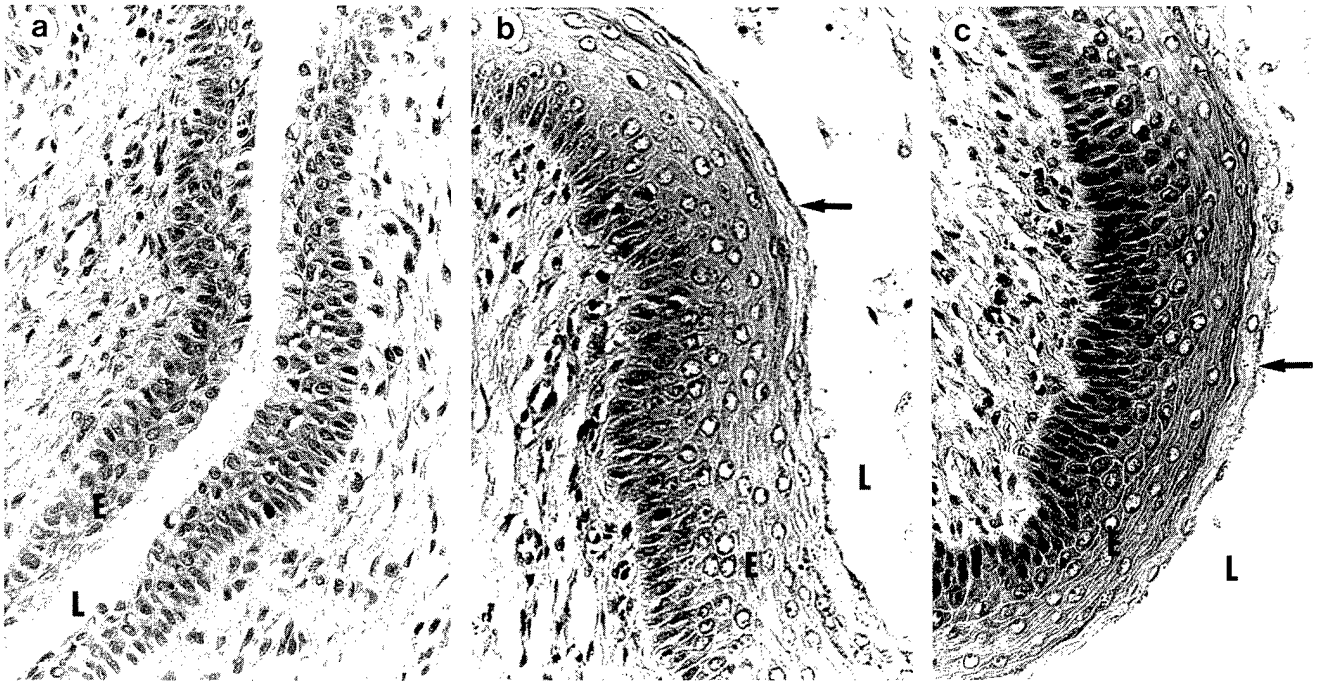

FIG. 1. Photomicrographs of vaginal epithelia. Rats were injected sc with olive oil or 10 μg/kg/day estradiol (E_2) on Days 21 and 22, or E_2 (Days 21 and 22) plus a pretreatment of TCDD (20, 40, or 80 μg/kg) on Day 19. All animals were euthanized on Day 23. Vaginal epithelium (E) from (a) a control rat, showing a transitional morphology; (b) an estradiol-treated rat, showing a stratified, squamous morphology and producing abundant amounts of keratin (arrow); or (c) a rat treated with estradiol plus 80 μg/kg TCDD, also showing a keratinizing epithelium. Results were confirmed in two separate experiments, for a total of eight animals per group (four animals in the 20 μg/kg TCDD dose group). L, vaginal lumen; 310× magnification.

drome (Seefeld *et al.*, 1984). In Sprague–Dawley rats, TCDD induces a wasting syndrome and significant lethality at 25 μg/kg (LD25) and 50 μg/kg (LD75) TCDD for a wide range of ages (Seefeld *et al.*, 1984; Christian *et al.*, 1986). In the present study, and as shown by others (Christian *et al.*, 1986), TCDD also caused a significant increase in liver weights at all dose levels tested (Table 1). Thymic weight, another well-documented target of TCDD toxicity (Poland and Knutson, 1982; Vos and Luster, 1989), was decreased to 68, 55, and 62% of control after exposure to 20, 40, and 80 μg/kg TCDD, respectively (Table 1). Estrogen alone had no effect upon either hepatic or thymic weight.

TABLE 2

Effects of Estradiol and TCDD on Uterine Estrogen Receptor and Progesterone Receptor Content and Serum FSH Levels[a]

Treatments	Estrogen receptor (fmol/mg protein)[b]	Progesterone receptor (fmol/mg protein)[c]	Serum FSH (ng/ml)[d]
Control	632.8 ± 164.6 (7)[e]	191.2 ± 24.8 (7)	6.8 + 2.4 (17)
Estradiol (E_2)	404.9 ± 63.8 (8)[f]	188.3 ± 86.7 (8)	4.1 + 1.1 (19)[g]
E_2 + 20 TCDD	472.9 ± 117.3 (4)	183.6 ± 29.5 (4)	3.7 + 1.0 (10)[g]
E_2 + 40 TCDD	418.3 ± 122.2 (7)[h]	193.9 ± 94.0 (7)	5.1 + 1.8 (18)[i]
E_2 + 80 TCDD	459.9 ± 99.1 (8)[h]	208.3 ± 83.7 (8)	4.3 + 1.3 (15)[g]

[a] Rats were treated as in Table 1. Dose levels of TCDD are in μg/kg.
[b] Estrogen receptor levels were analyzed by EIA, as discussed under Materials and Methods.
[c] Progesterone receptor levels were analyzed using a ligand binding assay.
[d] FSH serum levels were analyzed by RIA.
[e] All values are expressed as means ± standard deviations for (n) number of animals.
[f] Significantly different from control, $p = 0.003$.
[g] Significantly different from control, $p \leq 0.002$.
[h] Significantly different from control, $p < 0.03$.
[i] Significantly different from control, $p = 0.03$; significantly different from E_2 treated, $p = 0.043$.

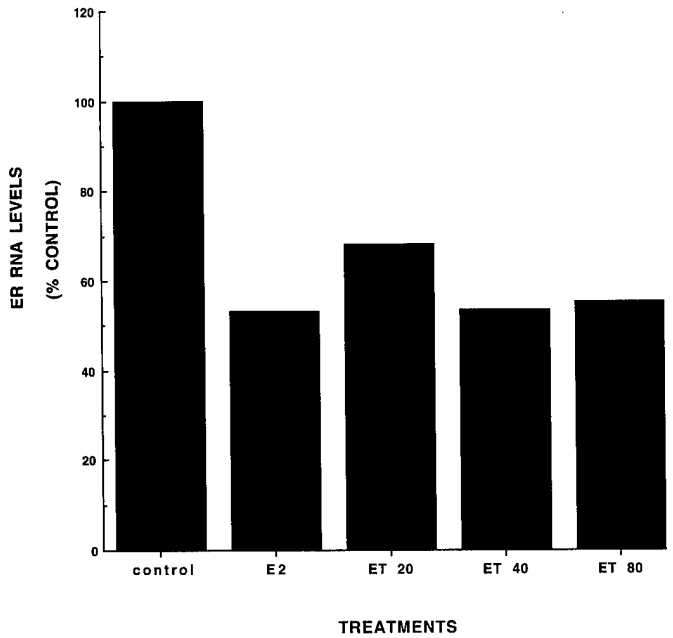

FIG. 2. Estrogen receptor mRNA levels. Animals were treated as in Fig. 1. Pairs of uteri within a treatment group were pooled, and total RNA was isolated, serially diluted onto a Nytran membrane, and analyzed using a slot blot hybridization procedure, as described under Materials and Methods. For each RNA sample, values were initially plotted as PhosphorImager units vs amount of RNA loaded. Mean PhosphorImager values for 10 μg total RNA were calculated from the linear portions of the curves. Values are expressed as percentages of control and represent the mean from two RNA samples per treatment from one experiment. Treatments: E2, estradiol alone; ET 20, estradiol + 20 μg/kg TCDD; ET 40, estradiol + 40 μg/kg TCDD; ET 80, estradiol + 80 μg/kg TCDD.

The induction of CYP1A1 was investigated as a sensitive biochemical index of TCDD exposure (Poland and Knutson, 1982). Using a specific CYP1A1 riboprobe, low basal levels of hepatic CYP1A1 mRNA were detected by slot blot analysis in control and E_2 exposure groups (Fig. 4). TCDD caused a dose-dependent induction of CYP1A1 message levels to 14, 20, and 30 times control levels at 20, 40, and 80 μg/kg TCDD, respectively.

DISCUSSION

The initial purpose of the present study was to establish a rat model for the reported antiestrogenic effects of TCDD (Romkes *et al.*, 1987; Astroff and Safe, 1988; Romkes and Safe, 1988) in order to elucidate an *in vivo* mechanism for these effects. In weanling female Sprague–Dawley rats, as expected, E_2 significantly increased uterine wet weight, caused vaginal keratinization, and decreased uterine ER protein and mRNA levels. Furthermore, E_2 significantly reduced circulating FSH levels. However, TCDD, at doses between 20 and 80 μg/kg, was not antiestrogenic in that it was unable to prevent any of these estrogen-dependent changes. TCDD also did not synergize with estrogen to enhance any responses to estrogen. In contrast, these doses of TCDD significantly decreased growth rates and thymic weights and caused liver hypertrophy, all indices of overt toxicity to TCDD (Poland and Knutson, 1982; Seefeld *et al.*, 1984; Vos and Luster, 1989). In addition, TCDD induced CYP1A1 gene expression in the liver to up to 30 times control levels, indicating that TCDD was being properly administered.

The results are in agreement with Shiverick and Muther (1982), who demonstrated that TCDD did not alter the uterotrophic response of ovariectomized female Holtzman rats to estrone exposure. Likewise, Hruska and Olson (1989) demonstrated that TCDD did not decrease, but rather increased, uterine ER content 7 days after exposure of immature (>29 days old) Sprague–Dawley rats to 50 μg/kg TCDD. In that study, TCDD also caused a reduction in body weight 7 days after treatment. Uterine ER content was also not changed in adult B6C3F1 mice after a 13-week chronic TCDD exposure, although the mice did show TCDD-dependent increases in CYP1A1 and CYP1A2 enzyme activities and increases in hepatic proteins containing phosphotyrosine residues (DeVito *et al.*, 1994).

On the other hand, our results are in contrast to several published reports in mice, rats, and other rodent species, showing an antiestrogenic effect of TCDD (Umbreit *et al.*, 1988; DeVito *et al.*, 1992a,b; Lin *et al.*, 1991; Romkes *et*

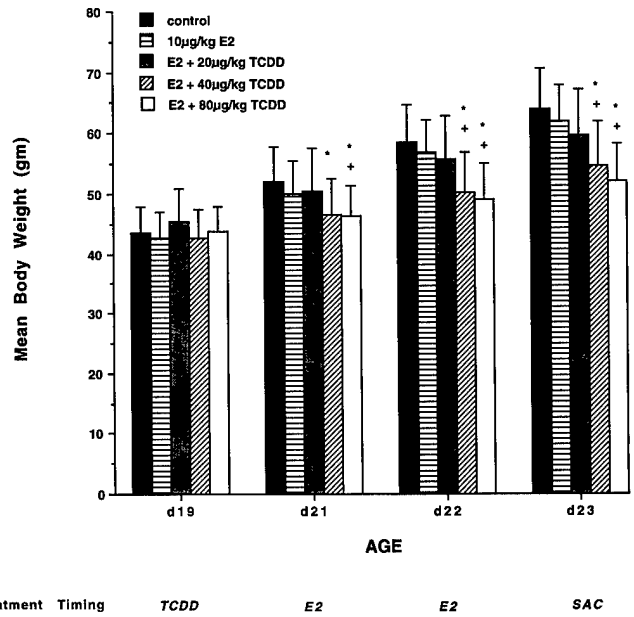

FIG. 3. Body weights of animals throughout the experiment. Animals were treated as in Fig. 1 and weighed on the day of each injection and at the time of euthanization. Days of injections with either E_2 or TCDD are indicated. The groups are control (solid black), estradiol alone (horizontal stripe), estradiol plus 20 μg/kg TCDD (ET20, solid gray), estradiol plus 40 μg/kg TCDD (ET40, diagonal stripe), and estradiol plus 80 μg/kg TCDD (ET80, solid white). *Significant difference from control, $p < 0.01$; +, significant difference from E_2 treatment, $p \leq 0.002$.

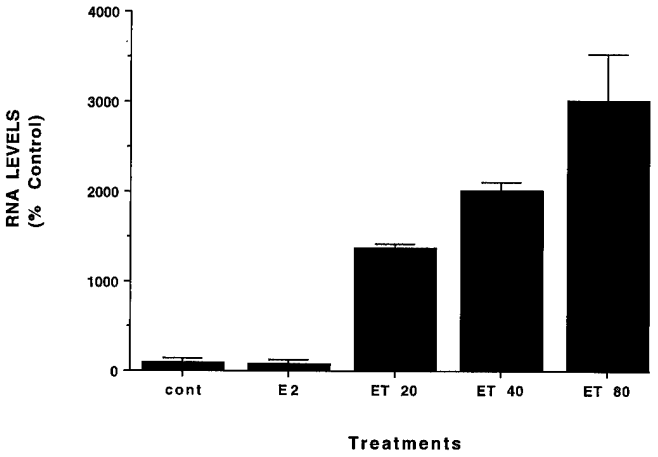

FIG. 4. CYPIA1 mRNA levels. Animals were treated as in Fig. 1. Total hepatic RNA was isolated from single liver samples, and slot blots were prepared as described under Materials and Methods. Mean PhosphorImager units were calculated as in Fig. 2. Values are expressed as percentages of control and represent the results of one experiment for a total of two samples per treatment group. Treatments: cont, control; E2, estradiol alone; ET 20, estradiol + 20 μg/kg TCDD; ET 40, estradiol + 40 μg/kg TCDD; ET 80, estradiol + 80 μg/kg TCDD.

al., 1987; Romkes and Safe, 1988; Astroff and Safe, 1988; Astroff *et al.*, 1990; Hruska and Olson, 1989). A plausible explanation for the discrepancy between those results and our own is that any antiestrogenic effects of TCDD are likely to be dependent upon species, strain, age, target tissue, and hormonal status. For example, Gallo, DeVito, and co-workers demonstrated that TCDD could antagonize E_2-dependent endometrial maturation and basal and E_2-stimulated uterine and hepatic ER levels in immature mice (Umbreit *et al.*, 1988; DeVito *et al.*, 1992a,b), whereas sexually mature mice were relatively insensitive to these effects (DeVito *et al.*, 1992c). The doses of TCDD used, although similar to those used in the present study, were well below the LD50 of TCDD in the CD-1 mouse and were not overtly toxic, establishing a *specific* antiestrogenic effect of TCDD in the mouse at relatively low doses.

Safe and co-workers have also reported that TCDD (20 and 80 μg/kg) can inhibit estrogen-dependent increases in uterine weight, uterine and hepatic ER levels, uterine PR levels, and uterine epidermal growth factor receptor activity and expression in Long–Evans and/or Sprague–Dawley rats (Romkes *et al.*, 1987; Romkes and Safe, 1988; Astroff and Safe, 1988; Astroff *et al.*, 1990). In those experiments, injections were generally administered to animals that were older than 25 days of age. In our preliminary studies with Long–Evans rats, a significant proportion of animals were already beginning to cycle by PND 27, and animals of this age were refractory to exogenously administered estrogen (data not shown). When younger Sprague–Dawley rats were employed (present study), they responded appropriately to E_2, but E_2-induced changes were not prevented by TCDD. Thus, age may play a crucial role in the ability of E_2 to elicit biological effects, as well as in the ability of TCDD to interfere with these effects in the rat, just as it does in the mouse. Hamsters, guinea pigs, and rats differ in their susceptibility to the antiestrogenic effects of TCDD (Hruska and Olson, 1989). Clearly there are also species differences between the mouse and the rat, since weanling mice are more sensitive to TCDD's antiestrogenic effects than adult mice, whereas weanling rats appear to be insensitive to these effects.

The age dependence of TCDD's antiestrogenicity implies that it is linked to the sexual maturation state of the endocrine system (Ojeda *et al.*, 1986). This might suggest that the antiestrogenicity of TCDD is regulated systemically. Central nervous system involvement has already been implicated in other systemic effects of TCDD. Body weight set point, which is, in part, controlled by the hypothalamus (Steffens and Strubbe, 1987), appears to be altered in the TCDD-induced wasting syndrome (Seefeld *et al.*, 1984). Hypophysectomy in mice leads to reductions in the LD50 of TCDD and the latency of mortality (DeVito *et al.*, 1992b). The pituitary/hypothalamic axis has also been implicated in TCDD's reproductive effects. For example, studies by Bookstaff and coworkers (1990) elegantly demonstrated that the male reproductive toxicity of TCDD was probably regulated at the level of the pituitary. Similarly, hypophysectomy in the female mouse lowered hepatic and uterine ER levels, but these levels were not further reduced by TCDD (DeVito *et al.*, 1992b). In addition, TCDD-exposed mice and monkeys and dibenzofuran-exposed women all showed cessation of estrous/menstrual cycling (Umbreit *et al.*, 1987; Barsotti *et al.*, 1979; Lu and Wong, 1984), again, implying a link between the antiestrogenic effects of TCDD and the pituitary/hypothalamic axis.

It can be argued that the exposure and euthanization regimen of the present study bypasses involvement of the pituitary/hypothalamic axis, thus allowing observations to be made on the direct effects of TCDD on estrogen target organs. E_2 was administered exogenously, rather than being released by the ovary under pituitary/hypothalamic control. In addition, the animals were euthanized 1 day after the second estrogen injection, and the large uterotrophic response (Hisaw, 1959), downregulation of ER (Medlock *et al.*, 1991), and lack of change in PR (Horwitz and McGuire, 1978) after E_2 exposure indicate that we were probably examining early direct effects of E_2 on E_2-responsive target organs. Finally, TCDD was administered as a pretreatment, allowing it to be available to block early estrogen-dependent changes. Under this paradigm, no effects of TCDD on estrogenic responses were observed. In contrast, other researchers examined estrogenic events 2 days after E_2 treatment (Romkes *et al.*, 1987; Romkes and Safe, 1988) and observed estrogen-induced responses, such as upregulation of ER, which may be secondary effects of E_2 (Medlock *et*

al., 1991). Likewise, a coadministration of TCDD and E_2, as used by other investigators (Romkes *et al.,* 1987; Romkes and Safe, 1988), would more likely result in TCDD-dependent effects on late, rather than early, E_2-dependent processes, since TCDD cannot directly interact with the ER (Romkes *et al.,* 1987). This, again, argues for an effect of TCDD on systemic processes, which could in turn mediate secondary effects of E_2 in target organs. In studies using mice (DeVito *et al.,* 1992a,b), E_2 was not administered exogenously. Therefore, pituitary/hypothalamic function was not bypassed in those studies, and antiestrogenic effects of TCDD were observed. Clearly, more studies are needed to definitively determine whether the pituitary/hypothalamic unit is involved in the antiestrogenic effects of TCDD. However, it is clear from our study that in the weanling rat, when estrogen is available to target tissues, TCDD cannot block early estrogen-dependent processes.

In the present study, the fact that TCDD produced overt toxic changes without altering estrogen-induced responses causes one to question the validity of ascribing a *specific* antiestrogenic ability to TCDD in the rat. Unlike the immature mouse, in which TCDD elicits antiestrogenic effects at doses which are more than an order of magnitude below the LD50 (DeVito *et al.,* 1992b), the antiestrogenicity of TCDD in Sprague–Dawley and Long–Evans rats (Astroff and Safe, 1988; Astroff *et al.,* 1990; Romkes *et al.,* 1987; Romkes and Safe, 1988) has only been reported at doses at or above the LD50 values for these strains (over a wide range of ages) (Seefeld *et al.,* 1984; Pohjanvirta *et al.,* 1993). TCDD is well known to cause a wasting syndrome, which is associated with decreased food intake (Poland and Knutson, 1982; Seefeld *et al.,* 1984; Christian *et al.,* 1986). Certainly, such systemic toxicity could lead to other secondary, homeostatic changes in the body, which could affect reproduction. For example, fasting alone is well-documented to elicit a state of anestrous (Knuth and Friesen, 1983). Since no body weights, thymic weights, or any other signs of morbidity were mentioned in the earlier studies of TCDD antiestrogenicity in the rat, it is impossible to know whether overt toxicity did occur in those animals. However, based on our results and those of others (Hruska and Olson, 1989), further studies which control for changes in body weight (e.g., pair feeding studies) are warranted to clarify the *specific* antiestrogenic potential of TCDD in the rat. Moreover, given the wide range of species sensitivity, age dependency, and possible involvement of the pituitary/hypothalamic axis in TCDD's antiestrogenic effects, and given the fact that TCDD cannot interact directly with the ER (Romkes *et al.,* 1987), TCDD might be more appropriately categorized as an endocrine disrupter, rather than an antiestrogen, per se.

In conclusion, TCDD did not show an antiestrogenic effect when administered to weanling female Sprague–Dawley rats, although it did produce a pattern of effects consistent with overt systemic toxicity. Clearly, antiestrogenicity is not a sensitive endpoint of TCDD action in these animals relative to other indices of toxicity, such as body weight and thymic weight. The antiestrogenic potential of TCDD is likely to be species, strain, age, target organ, and possibly hormonal status dependent. In any case, an antiestrogenic response in a particular species is probably not toxicologically relevant if it is only elicited at near-lethal doses. With this in mind, it will be interesting to determine which species show a *specific* antiestrogenic effect of TCDD, what the mechanism of that effect is, and whether or not TCDD's antiestrogenicity is sufficient to explain the reproductive effects of TCDD.

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Analysis of Structural Requirements for Ah Receptor Antagonist Activity: Ellipticines, Flavones, and Related Compounds

Thomas A. Gasiewicz,‡ Andrew S. Kende,† George Rucci,* Brenda Whitney† and J. Jeff Willey**

*Departments of Environmental Medicine and †Chemistry, University of Rochester, Rochester, NY 14642 U.S.A.

ABSTRACT. A number of studies have examined the structure-activity relationships for the agonist activity of Ah receptor (AhR) ligands. Fewer studies have considered the structural basis for potential antagonist properties. Certain ellipticine derivatives have been reported to bind to the AhR and inhibit the ability of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) to transform the AhR to a form that recognizes a dioxin-responsive enhancer element (DRE) upstream of the cytochrome P4501A1 gene. In the present study, over 30 ellipticine derivatives and structurally related compounds were examined for their ability to bind to the AhR, activate it to a DRE-binding form, induce the luciferase gene under control of a DRE-containing enhancer, and block activation of the AhR by TCDD. The ability of several ellipticine derivatives to inhibit TCDD-elicited DRE binding and TCDD-induced luciferase activity was inversely related to their ability to alone stimulate these responses. The most potent antagonist activity was related to good AhR binding characteristics in terms of conforming to previously predicted 14 × 12 × 5 Å van der Waals dimensions and the presence of an electron-rich ring nitrogen at or near a relatively unsubstituted X-axis terminal position. Based on these data, a number of flavone derivatives were synthesized and tested for their relative agonist/antagonist activity. These additional data were consistent with the hypothesis that an electron-rich center near or along a lateral position of the van der Waals binding cavity is a characteristic that enhances AhR antagonist activity. *Copyright © 1996 Elsevier Science Inc.* BIOCHEM PHARMACOL 52;11:1787–1803, 1996.

KEY WORDS. ellipticines; flavones; Ah receptor binding; antagonism

The AhR is a transcription factor and member of the bHLH class of DNA-binding proteins. Within this class is a subgroup of proteins termed the PAS family based on their domain and sequence similarities [1–3]. The PAS members include Arnt [4], a dimerization partner for the AhR, two *Drosophila* proteins Per and Sim [3], and hypoxia-inducible factor 1-α [5]. The AhR is the only member of the bHLH class known to be activated by a ligand. In the absence of ligand the AhR is localized in the cytosol associated with hsp90 [6] and possibly other proteins [7]. Agonist binding initiates a series of as yet undefined events resulting in hsp90 dissociation, nuclear localization, and heterodimerization with the Arnt protein [4, 8–10]. The resulting complex is able to interact with *cis*-acting elements, DREs, found within the 5′ regulatory regions of responsive genes to modulate rates of transcription [2]. Ligand-elicited activation of the AhR has been shown to alter the transcription of a number of genes, including several oncogenes and those encoding growth factors, in a tissue- and species-specific manner [11]. However, only a few of these are known to contain functional DREs [12–16].

A variety of xenobiotics have been shown to bind to the AhR and act as receptor agonists to elicit the prototypical AhR-mediated biochemical responses such as induction of *CYP1A1* [12]. These agonists include certain halogenated biphenyls, terphenyls, dibenzofurans, and dibenzo-*p*-dioxins. Of these, TCDD is the most potent and well-studied congener [2, 17]. The exposure of mammals to these xenobiotics elicits a variety of species-specific biologic and toxic effects including thymic atrophy and immune suppression, developmental and reproductive alterations, and tumor promotion [11]. It has been postulated that the inappropriate induction and/or repression of genes regulated by the AhR affects cellular differentiation states, and this leads to the toxic consequences observed. The types of biological effects elicited, together with the biochemical and functional characteristics of the AhR, suggest that this protein likely has some normal function that may be regu-

‡ Corresponding author. Tel. (716)275-7723; FAX (716)256-2591.

§ *Abbreviations:* ANF, alpha-naphthoflavone; AhR, Ah receptor; ARNT, Ah receptor nuclear translocator, bHLH, basic helix-loop-helix; CYP1A1, cytochrome P4501A1; DRE, dioxin-responsive element; EMSA; electrophoretic mobility shift assay; and TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

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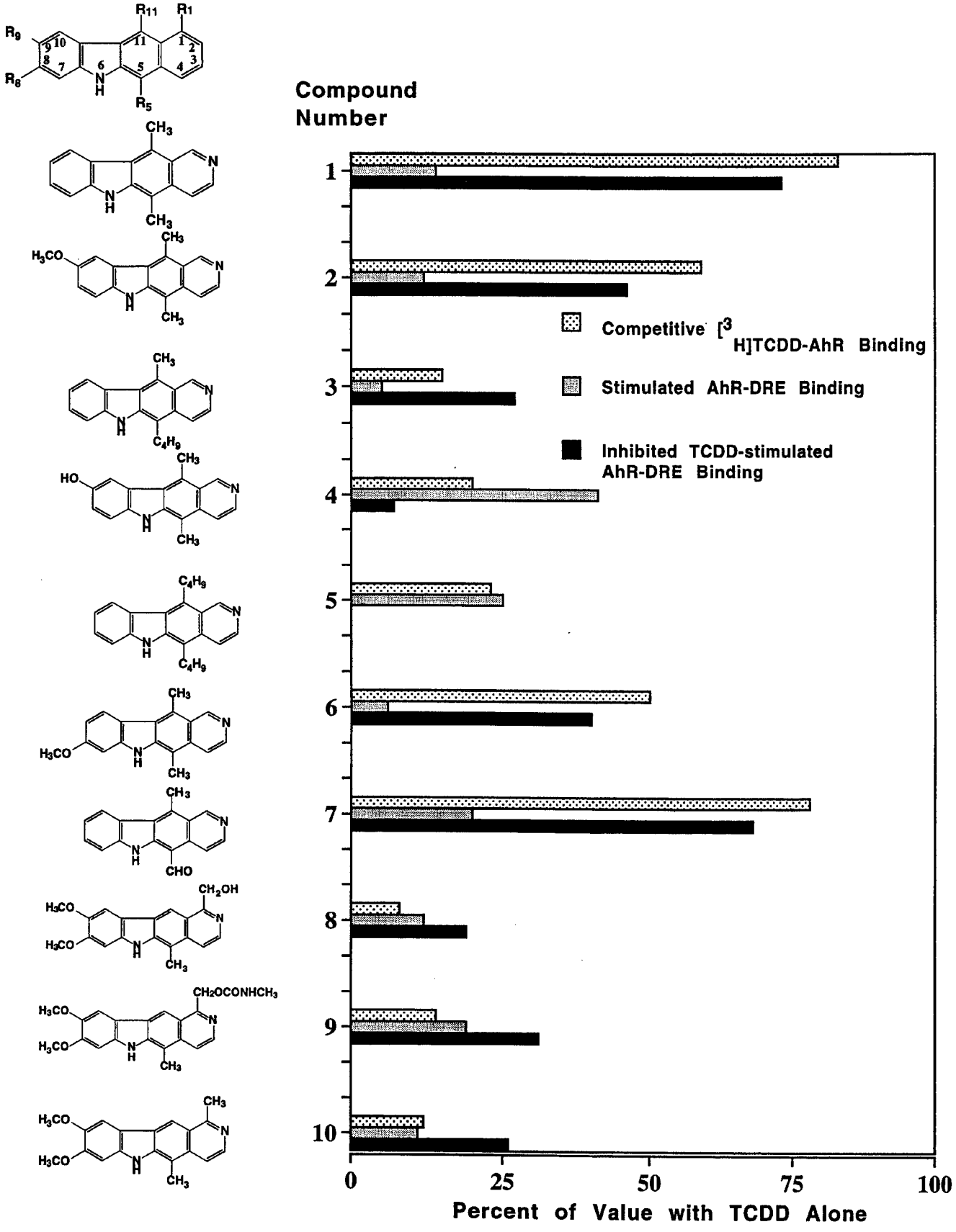

Compound Number
1
2
3
4
5
6
7
8
9
10
Competitive [3H]TCDD-AhR Binding
Stimulated AhR-DRE Binding
Inhibited TCDD-stimulated AhR-DRE Binding
0
25
50
75
100
Percent of Value with TCDD Alone

FIG. 1.

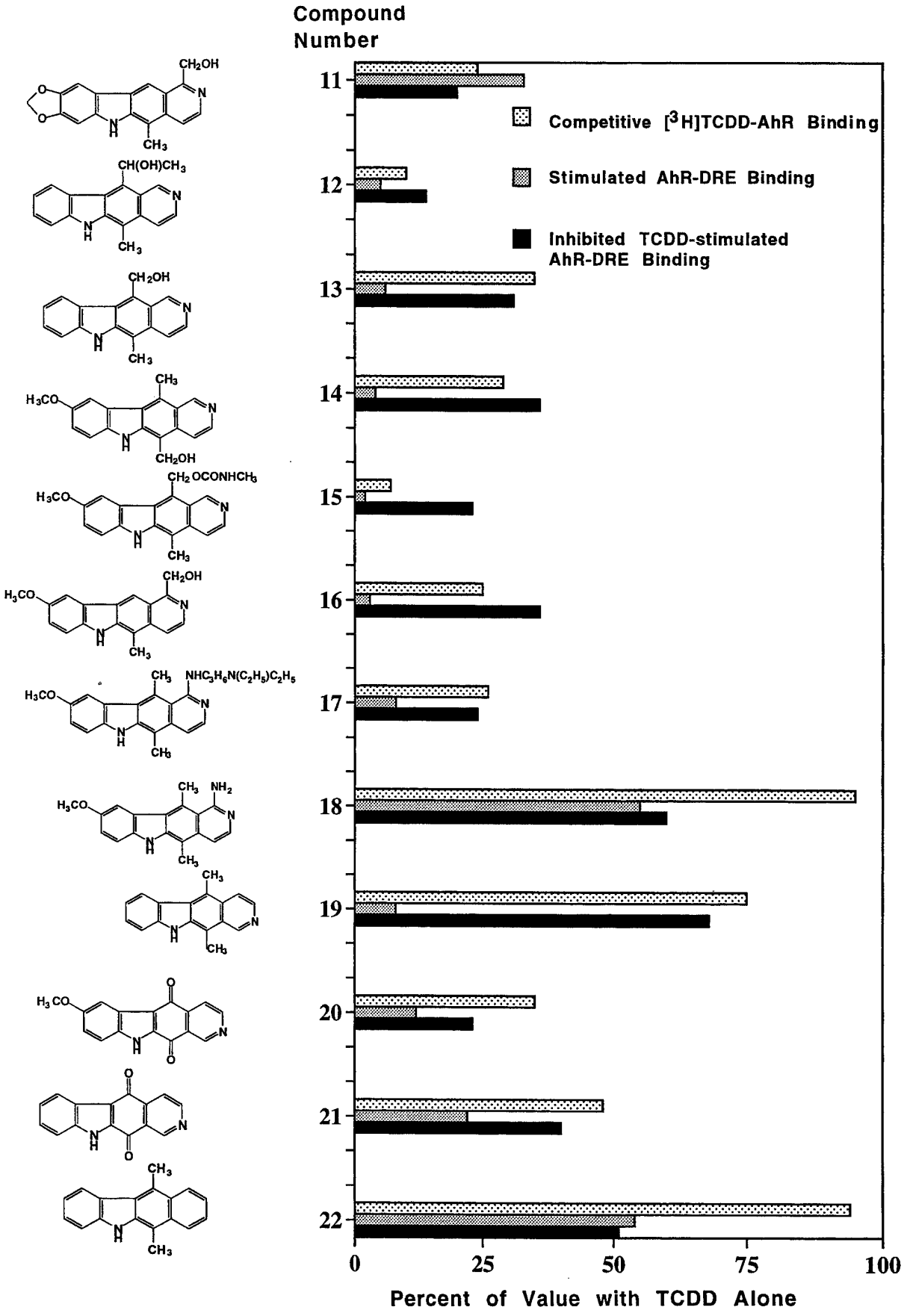


FIG. 1. Continued.

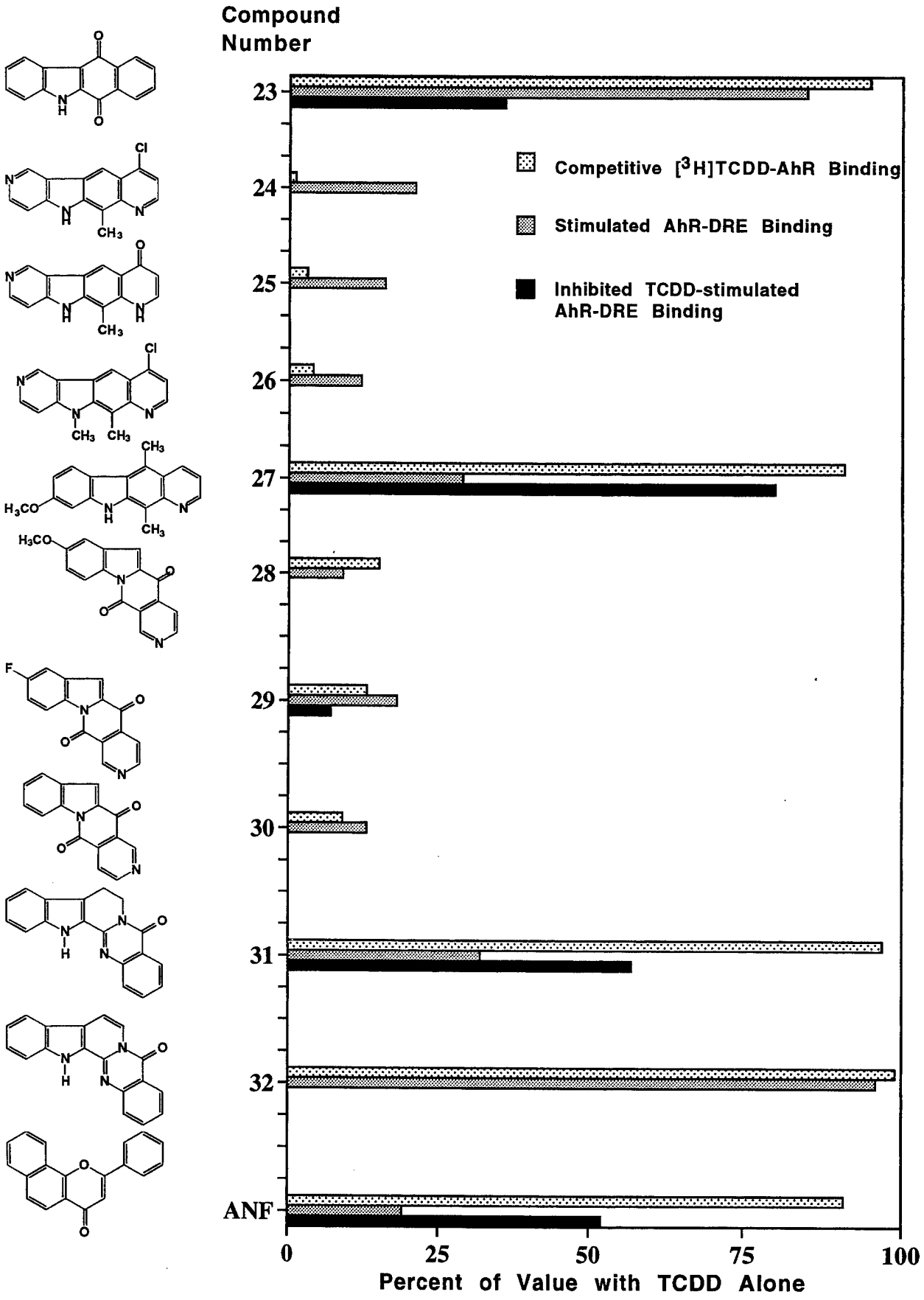


FIG. 1. Continued.

lated by an endogenous ligand. Recent work describing the characteristics of a mouse strain in which a functional AhR protein has been deleted suggests a role in the development of the liver and immune system [18]. The specifics of this role and an endogenous ligand have yet to be identified.

One approach to questions involving the function of the AhR and the role of ligands in regulating this function is to determine how changes in ligand structure alter activity of the protein. A large number of studies have focused on the structure-activity relationships for agonist activity of potential ligands [e.g. 19–22]. However, fewer studies have considered the structural basis for potential antagonist properties. Chemicals such as 2,2′,4,4′,5,5′-hexachlorobiphenyl [23], 1-amino-3,7,8-trichlorodibenzo-*p*-dioxin [24], and 6-methyl-1,3,8-trichlorodibenzofuran [25] have been reported to antagonize certain responses elicited by TCDD. However, these compounds can be best categorized as only partial and/or weak antagonists; ANF (7,8-benzoflavone), an isomer of the AhR agonist β-naphthoflavone (5,6-benzoflavone), binds to the AhR and antagonizes TCDD-mediated *CYP1A1* induction and immunosuppression [26]. However, ANF has agonist properties at higher concentrations [27]. Likewise, other compounds such as certain ellipticines [28, 29] and phenanthrolines [30] either have a comparatively lower affinity for the AhR or are also partial antagonists.

It was of interest to determine whether there are any structural similarities among compounds that possess antagonist properties. Analogs of ellipticine have been used by the European medical community for the treatment of breast cancer. The anti-neoplastic effects of these compounds are attributed to their activity as topoisomerase II inhibitors [31, 32]. However, certain ellipticine derivatives also have been found to bind to the AhR and inhibit the ability of TCDD to elicit the transformation of the AhR to a DRE-binding state [28, 29]. In the present study, over 30 ellipticine derivatives and structurally-related compounds were analyzed for their relative ability to bind to the AhR. In addition, their relative agonist/antagonist activity was determined by their ability to activate/inhibit the AhR to a DRE-binding form and induce/block a reporter gene under control of a DRE-containing enhancer. Data from these studies were used to arrive at a hypothesis regarding structural characteristics for compounds with antagonist activity. Based on this hypothesis, flavone derivatives were synthesized and tested for their relative agonist/antagonist activity. These combined data indicated consistent structural characteristics that contribute to antagonist activity.

MATERIALS AND METHODS

Chemicals

Ellipticine derivatives and related compounds (Fig. 1) were provided by Drs. C. Rivalle and E. Bisagni (Institut Curie, France), S. Archer (Rensselaer Polytechnic Institute, Troy, NY, U.S.A.), V. Milata (Slovenska Technicka Univerzita, Bratislava, Slovenia), and G. W. Gribble (Dartmouth College, Hanover, NH, U.S.A.). The rutaecarpine alkaloids (compounds **31** and **32**; Fig. 1 and Table 1) were provided by Dr. J. Bergman (Royal Institute of Technology, Stockholm, Sweden). 1-Amino-3,7,8-trichlorodibenzo-*p*-dioxin was obtained from Dr. M. Luster (NIEHS, Research Triangle Park, NC, U.S.A.). The 7,8-benzoflavone derivatives (Table 2) were synthesized as described by Doyle *et al.* [33] and Nowlan *et al.* [34]. 3′-Methoxy-4′-nitroflavone, 3′-methoxy-4′-aminoflavone, and 4′-nitroflavone (Table 2) were synthesized by the procedure of Cunningham *et al.* [35]. The structures and purities (>98%) of the synthesized compounds were confirmed by nuclear magnetic resonance spectroscopy and determination of melting point. [^{3}H]TCDD (40 Ci/mmol) was obtained from Chemsyn Science Laboratories (Lenexa, KS, U.S.A.), and unlabeled TCDD was obtained from Cambridge Isotopes (Cambridge, MA, U.S.A.). [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from NEN Research Products (Boston, MA, U.S.A.). All of the other chemicals and biochemicals were of the highest purity commercially available.

Buffers

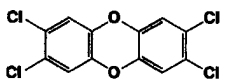
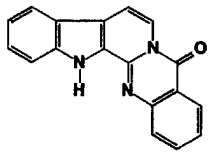
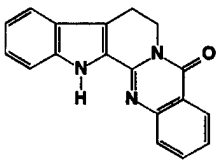
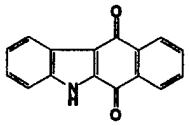
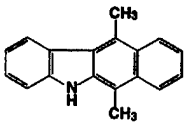
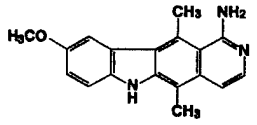
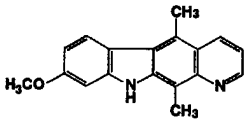
HEDG buffer consisted of 25 mM HEPES, 1.5 mM EDTA, 1 mM dithiothreitol, and 10% (v/v) glycerol, pH adjusted to 7.6 at room temperature.

Animals and Cytosol Preparation

Male Sprague–Dawley rats (250–300 g; Charles River, Wilmington, MA, U.S.A.) were housed with a 12-hr light-dark cycle and were allowed food and water *ad lib*. Animals were killed by CO_2 overdose and livers were perfused with HEDG plus 1.15% KCl. Cytosol was prepared as described

FIG. 1. Competitive binding of 1 μM ellipticine derivatives and related compounds to rat hepatic AhR, and their activity to alone stimulate or inhibit TCDD-stimulated AhR binding to the DRE. For competition studies, rat hepatic cytosol was incubated with 3 nM [^{3}H]TCDD + 1 μM competitor for 2 hr at 20°. Specific binding was determined by the hydroxylapatite assay. Values are expressed as percent inhibition of [^{3}H]TCDD specific binding and are the means of duplicate or triplicate analyses. Variation from the mean was <10% in all cases, and the average absolute value for the specific binding of [^{3}H]-TCDD was 2593 dpm/35 μL of cytosol. To determine the effects on AhR-DRE binding, rat hepatic cytosol was incubated with 1 μM test compound ± 3 nM TCDD and EMSA was performed as described in Materials and Methods and Fig. 2 legend. Quantitation was performed by PhosphorImager analysis. Values are expressed as percent of the response produced by 3 nM TCDD alone. The absolute value for TCDD varied considerably with the time of screen exposure, but averaged 365,606 phosphor units per 15 μL of cytosol loaded per lane. The absence of data for the inhibition of TCDD-elicited DRE binding for compounds 5, 24, 25, 26, 28, 30, and 32, indicates that no inhibition was detected at the concentration used.

TABLE 1. Competitive binding of selected ellipticine derivatives and related compounds to rat hepatic AhR, and their ability to alone stimulate or inhibit TCDD-elicited transformation of the AhR to a DRE-binding form

Chemical structure	Compound designation	Competitive AhR binding		Stimulated DRE binding At 1 μM†	Inhibition of TCDD-elicited DRE binding	IC_{50} (DRE) Binding / IC_{50}(AhR Binding)*
		IC_{50} (nM)‡	K_i (nM)‡	%	IC_{50} (nM)‡	
	TCDD	3.5	0.12§	100	—	—
	32	4.9	0.17	96	>1000	>208
	31	25	0.86	32	192	7.6
	23	40	1.4	85	>1000	>25
	22	93	3.2	54	831	8.9
	18	135	4.6	55	550	4.1
	27	159	5.4	29	257	1.6

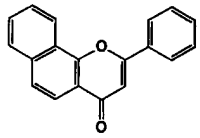
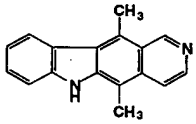
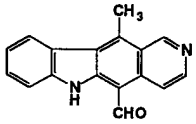
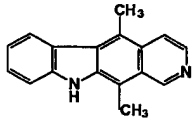
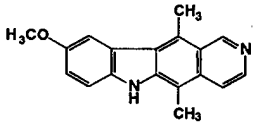
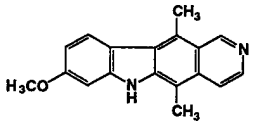
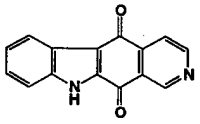
(continued)

previously [36] and frozen at −80° until use. Prior to use, cytosol was adjusted to 15 mg protein/mL. Protein concentrations were measured using the method of Waddell [37] with BSA as a standard.

Incubations and Competitive Ligand Binding Assay

Compounds were assayed for their ability to compete with [^{3}H]TCDD (3 nM) for AhR binding using the hydroxylapatite assay essentially as described previously [38]. Various concentrations (.001 to 1.0 μM) of competitor were added simultaneously with [^{3}H]TCDD to the cytosol and the incubation was performed at room temperature (approximately 20°) for 2 hr. Although equilibrium binding conditions were not specifically determined for each compound used in the present investigation due to their limited supply, the conditions used were previously found to achieve equilibrium binding of [^{3}H]TCDD even in the presence of a variety of competitors including the partial antagonist ANF [38–40]. In addition, the binding affinities we obtained for the rutaecarpines were similar to those reported by others [41]. Specific binding was determined as the difference between total binding ([^{3}H]TCDD alone) and nonspecific binding ([^{3}H]TCDD + 150-fold excess of 2,3,7,8-tetrachlorodibenzofuran). Values shown are expressed as percent inhibition of [^{3}H]TCDD specific binding, and are the results of duplicate or triplicate determinations. In all cases, variation from the mean was less than 10%. IC_{50} Values are defined as the concentration required to inhibit by 50% the binding of [^{3}H]TCDD to the AhR. These values were determined by nonlinear regression analysis of the binding data assuming 100% inhibition as the maximal

TABLE 1. Continued

Chemical structure	Compound designation	Competitive AhR binding		Stimulated DRE binding At 1 μM†	Inhibition of TCDD-elicited DRE binding	IC_{50} (DRE) Binding / IC_{50}(AhR Binding)*
		IC_{50} (nM)‡	K_i (nM)‡	%	IC_{50} (nM)‡	
	ANF	226	7.7	19	380	1.7
	1	272	9.3	14	270	1.0
	7	285	9.8	20	316	1.1
	19	358	12.3	8	478	1.3
	2	858	29.4	12	~1550	~1.8
	6	~1031	~35.3	12	~2000	~1.9
	21	~1088	37.3	22	~2000	~1.8

* The ratio of the IC_{50} value for the inhibition of TCDD-stimulated DRE binding (fifth column) to the IC_{50} value for the competitive binding to the AhR (second column).

† Values as percent of the DRE-binding activity produced by 3 nM TCDD. A 1 μM concentration of the particular compound was used.

‡ IC_{50} Values for competitive binding and inhibition of TCDD-elicited DRE-binding were determined using 3 nM TCDD and .001 to 1.0 μM concentrations of competitor as described in Materials and Methods. K_i Values were calculated from the IC_{50} values. Results are the mean of duplicate or triplicate determinations.

§ K_d as determined in Gasiewicz and Rucci [40].

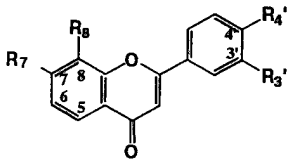
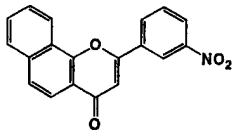
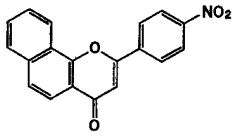
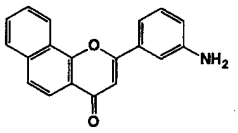
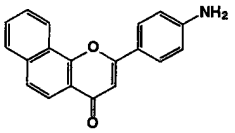
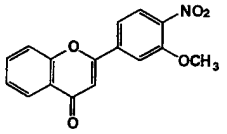
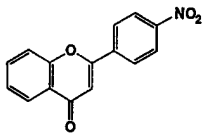
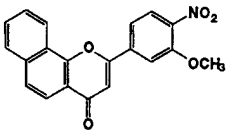
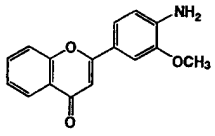
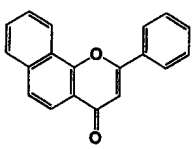
inhibition and a first-order receptor-binding model in which A = B − B/(1 × (C/D)^e) where A = fractional inhibition, B = the fractional maximal inhibition (=1.0), C = concentration of inhibitor (in log units), D = concentration of inhibitor that produces 50% inhibition (in log units), and e = the slope of the function. The analysis was performed using the Statistica™ software package from StatSoft (Tulsa, OK). K_i Values were calculated by the method described by Cheng and Prusoff [42] using a determined K_d value for unlabeled TCDD of 0.12 nM* [39].

* At infinite dilution, the affinity of the AhR for TCDD is estimated to be 7–10 pM [43].

Assessment of DRE Binding (EMSA)

The complementary oligodeoxynucleotides 5′-GATCCGGCTCTTCTCACGCAACTCCGAGCTC-A-3′ (noncoding strand) and 5′-GATCTGAGCTCG-GAGTTGCGTGAGAAGAGCCG-3′ (coding strand) were synthesized (Bio–Synthesis, Lewisville, TX, U.S.A.) and ^{32}P-labeled at the 5′ ends using T4 polynucleotide kinase and [γ-^{32}P]ATP. The annealed oligonucleotide contains a single core recognition sequence (underlined) for the DRE-binding form of the AhR [12]. To determine transformation of the AhR to the DRE-binding state, rat hepatic cytosol was incubated with either 3 nM TCDD, test compound, or TCDD plus various concentrations of the

TABLE 2. Competitive binding of flavone derivatives to rat hepatic AhR, and their ability to alone stimulate or inhibit TCDD-elicited transformation of the AhR to a DRE-binding form

Chemical structure	Compound designation	Competitive AhR binding		Stimulated DRE binding At 1 μM†	Inhibition of TCDD-elicited DRE binding	IC_{50} (DRE) Binding / IC_{50}(AhR Binding)*
		IC_{50} (nM)‡	K_i (nM)‡	%	IC_{50} (nM)‡	
	Parent Compound	—	—	—	—	—
	33	909	31.1	2	820	0.90
	34	419	14.4	20	460	1.1
	35	940	32.2	5	~1200	~1.3
	36	⩾1000	>34	0	⩾1000	—
	37	35	1.2	7	38	1.1
	38	>1000	>34	0	>1000	—
	39	10	0.34	30	22	2.2
	40	459	16	8	655	1.4
	ANF	226	7.7	19	380	1.7

* The ratio of the IC_{50} value for the inhibition of TCDD- stimulated DRE binding (fifth column) to the IC_{50} value for the competitive binding to the AhR (second column).

† Values as percent of the DRE-binding activity produced by 3 nM TCDD.

‡ IC_{50} Values for competitive binding and inhibition of TCDD-elicited DRE binding were determined using 3 nM TCDD and various concentrations of flavones as described in Materials and Methods. K_i Values were calculated from the IC_{50} values. Results are the mean of duplicate or triplicate determinations. Some numbers are designated as approximations due to extrapolation of the log-logit plots.

test compound for 2 hr at 20°. Aliquots containing 60–90 μg protein were incubated for 20 min at room temperature with 25,000–35,000 cpm of the ^{32}P-labeled DRE-containing oligonucleotide, 200 ng herring sperm DNA (Sigma Chemical Co., St. Louis, MO), and 0.08 M NaCl, followed by non-denaturing gel electrophoresis and autoradiography as described previously [12]. Autoradiographs are shown only for TCDD and compound **27.** The AhR-bound ^{32}P-labeled oligonucleotide bands were quantitated using a Model PSI PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.). The amount of radioactivity present at the same position in a non-ligand-treated lane was subtracted as background. Values indicated are as percent of the ArR-DRE binding produced by TCDD alone. IC_{50} Values were approximated from the log-logit plots of the inhibition data.

Reporter Plasmid

The reporter plasmid p2Dluc was constructed by inserting a fragment containing two DREs (DRE_D (sequence shown above) as designated by Lusska *et al.* [44], and a minimal promoter into the *Xho*I-*Hind*III site of the luciferase (luc) reporter vector, pGL2-Basic Vector (Promega, Madison, WI, U.S.A.). Enzymes were purchased from Boehringer Mannheim (Indianapolis, IN, U.S.A.). The initial minimal promoter construct, p2DlovTATA (made and provided by Dr. T. Kent, University of Rochester), was made by ligating the same 32-b oligonucleotides used for the DRE-binding analysis (described above) to form multimers. Protruding ends were filled in with *Taq* polymerase (100 nM dNTPs, 2 mM $MgCl_2$, 72°, 1 hr) before ligation into pCRII vector (Invitrogen, San Diego, CA, U.S.A.). Inserts that contained 2 DREs were digested with *Eco*RI, and cloned into plovTATA [45] (provided by Dr. J. Stein, SUNY, Syracuse, NY, U.S.A.), which contains a TATA box from the chicken ovalbumin promoter. PCR primers were synthesized that flanked the *Eco*RI site and TATA box and contained new restriction sites for *Xho*I and *Hind*III. These primers, 5′-CTAAAGCTTCTCGGGGAATATATA-3′ and 5′-GTACTCGAGTTTCCCAGTCACGAC-3′, were used to amplify this region from p2DlovTATA or plovTATA. The PCR product was digested with *Xho*I and *Hind*III and cloned into pGL2-Basic Vector, creating pTATAluc and p2Dluc, with zero and 2 DREs, respectively.

Cell Culture and Transfections

Hepa 1c1c7 cells were kept in an atmosphere of 5% CO_2 in minimum essential medium (MEM, from Sigma) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, U.S.A.). For transient transfections, cells were seeded at a density of 2×10^7 cells per 150 × 25-mm plate and incubated for 16 hr, after which time the medium was removed. Fresh medium was added and the cells were allowed to incubate for an additional 3 hr. Plasmid suspensions of pTATAluc or p2Dluc were prepared in 0.13 M $CaCl_2$ and HBSS, and 33.8 μg of plasmid DNA was added to each plate followed by a 4-hr incubation. Following removal of the medium, cells were glycerol-shocked (2.25 mL of 15% glycerol in HBSS) for 2 min. Each plate was washed twice with HBSS, medium was added, and the cells were incubated for 30 min. Cells contained in a number of plates were trypsinized, pooled, resuspended in medium, and distributed in aliquots to 60 × 15-mm plates in a total volume of 5 mL. Cells from each 150 × 25-mm plate were used to seed six 60 × 10-mm plates. These latter procedures were performed to control for differing levels of transfection efficiency. These cells were allowed to attach and grow overnight prior to treatment with the test chemicals.

Ligand-Mediated Luciferase Induction

Each of the chemicals was separately dissolved in DMSO and mixed in cell culture medium. Each of 3 plates was treated with chemical in 5 mL of medium (containing 2.5 μL DMSO). In cases where cells were treated with both TCDD and test chemical, the chemical was added approximately 30 min prior to TCDD (0.5 nM) treatment. Cells were incubated for 8 hr, harvested by manual scraping in calcium- and magnesium-free HBSS, and pelleted by centrifugation at 1000*g* for 2 min. The Luciferase Assay System (Promega) was used to determine luciferase activity. Cells from separate plates were resuspended in 60 μL of Reporter Lysis Buffer, and 10–20 μL was taken for analysis. Light units were determined by a luminometer (Turner Model TD-20e; Turner Designs, Sunnyvale, CA). The data are presented as percent of the activity observed in the presence of TCDD alone. IC_{50} Values were approximated from the log-logit plots of the inhibition data obtained using a range of concentrations of the compound.

Molecular Modeling and Analysis

Modeling and analysis of chemical structures was performed using the CSC ChemOffice™ program purchased from Cambridge Scientific Computing, Inc. (Cambridge, MA).

RESULTS

Ellipticines

We initially examined for the ability of concentrations up to 1 μM of each ellipticine derivative and structurally related compound to (1) compete with [^{3}H]TCDD for binding to the rat hepatic AhR, (2) alone elicit DRE-binding of the AhR, and (3) inhibit TCDD-elicited DRE-binding. We also examined two rutaecarpine alkaloids for which some data had been previously published [41]. The chemical structures of the compounds examined and these data obtained under cell-free conditions are presented together in Fig. 1. Those compounds that at 1 μM inhibited >50% of [^{3}H]TCDD binding were more thoroughly examined for

their agonist/antagonist characteristics under both cell-free and whole cell conditions. While much of the data in this paper focuses on these latter compounds and a number of flavones, for a thorough consideration of structure-activity relationships it is useful to present and consider the structural features of all compounds tested that did *and* did not show good affinity for the AhR.

In agreement with previous investigations [41], rutaecarpine and 7,8-dehydrorutaecarpine (compounds **31** and **32**, respectively; Fig. (1) demonstrated a high degree of competitive displacement. The ellipticine derivatives showed considerable variability in binding, with most of these competing less than 50% of [^{3}H]TCDD binding. Nevertheless, some consistent features were observed. Those compounds with large substituent groups at the 1, 5, or 11 positions demonstrated lower affinity, while those derivatives that were relatively unsubstituted or substituted with small groups, i.e. methyl, methoxy, or carbonyl oxygen, showed the highest affinity. The absence of the ring nitrogen at the 2 or 3 positions slightly enhanced the binding affinity (compare **22** with **1** (ellipticine) and **19**; **23** with **21**). The amino group at the 1 position also enhanced the affinity (compare **2** and **18**). Many of those compounds having the highest affinity (displacement of >50% at 1 μM) had IC_{50} and K_i values within an order of magnitude of that determined for ANF, but over 10-fold higher than that for TCDD (Table 1).

Heterodimerization of the agonist-bound AhR with Arnt and their binding to the DREs present in the 5′ regulatory region of the *CYP1A1* gene are necessary for TCDD-induced transcription of this gene [2]. As a measure of *potential* agonist activity, the ability of ellipticine and related compounds to alone elicit transformation of the AhR to a DRE-binding form was examined using EMSA. As previously shown by a number of investigators, EMSA of rat hepatic cytosol incubated with and without TCDD indicated the presence of a single TCDD-dependent protein-DRE complex (Fig. 2). Many of the compounds e.g. compound **27** (Fig. 2A), produced a concentration-dependent increase in a band having the same mobility as that elicited

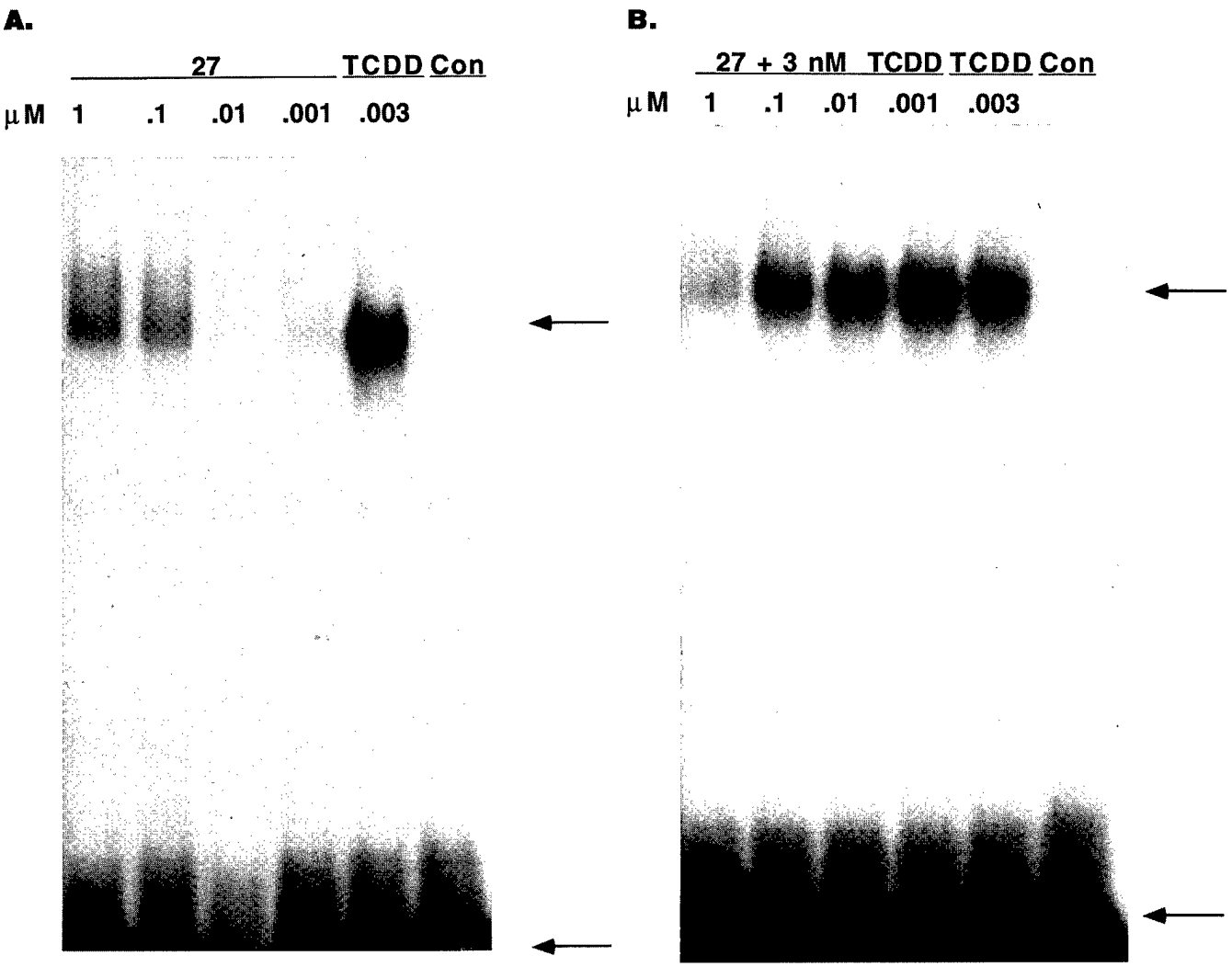


FIG. 2. EMSA of cytosol incubated with TCDD (3 nM) or compound 27 (A), or TCDD in the absence or presence of compound 27 (B). Rat hepatic cytosol was incubated with the indicated concentrations of ligand for 2 hr, 20° followed by EMSA as described in Materials and Methods. Top arrow indicates the AhR-bound ^{32}P-labeled DNA. Bottom arrow indicates the free DNA. Con (control) indicates cytosol incubated in the presence of vehicle (*p*-dioxane; 10 μL/mL).

by TCDD. However, there was much variability in the relative potency of these compounds to produce this response (Fig. 1). At a concentration of 1 μM, only compounds **18, 22, 23,** and **32** transformed the AhR to greater than 50% that observed using 3 nM TCDD. The other compounds tested that showed high affinity for the AhR demonstrated a much weaker ability to transform the AhR to a DRE-binding state (Table 1). Compound **31,** despite having an apparent affinity similar to compound **23,** elicited much less DRE-binding activity. Likewise, compound **27** showed considerably weaker AhR-DRE-binding activity as compared to compound **18.** Similarly, compounds **1, 2, 6, 7** and **19** demonstrated much less AhR-DRE-binding activity than what might otherwise be expected from their relative affinities. For example, although compound **1,** at a concentration of 1 μM, excluded approximately 83% of the [^{3}H]TCDD from binding to the AhR, this same concentration elicited only 15% of the DRE-binding activity as did TCDD.

A comparison of the elicited DRE-binding activity, as a percent of the response elicited by TCDD alone, with the percent inhibition of [^{3}H]TCDD-AhR binding produced at a competitor concentration of 1 μM (Fig. 1) appeared to give a useful reflection of the potential antagonist properties. All of the ellipticine derivatives listed that showed a comparatively weaker ability to elicit a DRE-binding conformation were relatively unsubstituted and had an electron-rich ring nitrogen at either the 2 or 3 positions (e.g. compare compound **22** with **1** and **19**). An exception to this pattern was compound **4** (9-hydroxyellipticine) which elicited significant DRE-binding activity at a concentration of 1 μM despite its relatively low affinity for the AhR. This might be related to the reported ability of this compound to activate protein kinase C at this concentration (although it is inhibitory at higher concentrations) [29], in conjunction with the reported involvement of this enzyme in the transformation of the AhR to a DNA-binding form [46]. In the case of compound **18,** the presence of the amino group at position 1 appeared to increase affinity for the AhR, but negated the inhibitory effect of the adjacent electron-rich ring nitrogen (compare **2** with **18**).

The ability of these compounds to inhibit TCDD-elicited transformation of the AhR to a DRE-binding state was determined. None of the compounds completely blocked DRE-binding of the TCDD-AhR complex at a competitor concentration of 1 μM (Fig. 1). Compound **27** demonstrated the greatest inhibitory activity (Fig. 2B). Of those compounds that had the highest affinity for the receptor (Table 1), there was a consistent inverse relationship between the ability to alone stimulate DRE binding and inhibit TCDD-induced DRE binding. Compound **32,** which exhibited the highest receptor affinity and the greatest activity in terms of stimulating DRE binding, produced no inhibition of TCDD-induced AhR-DRE binding at a concentration of 1 μM (Fig. 1). In contrast, compounds **1, 7, 19,** and **27,** all of which showed very weak ability to stimulate DRE binding, demonstrated the strongest ability to inhibit TCDD-induced activity. The IC_{50} values for the inhibition of DRE binding by these compounds were very close to the IC_{50} values for the competition of [^{3}H]TCDD for AhR binding (see the IC_{50}-DRE binding/IC_{50}-AhR binding ratios in Table 1). Likewise, compounds **2, 6, 21, 22,** and **31** exhibited a relatively good ability to inhibit the TCDD-stimulated DRE binding as indicated by the IC_{50}-DRE binding/IC_{50}-AhR binding ratios.

Based on the above data along with that of other compounds known to posses partial AhR antagonist activity i.e. certain phenanthrolines [30], we proposed a hypothesis to identify structural requirements for the best antagonist activity, as operationally defined by the ability to inhibit TCDD-elicited DRE-binding of the AhR. Firstly, the compounds should fit the hypothetical 14 × 12 × 5 Å van der Waals receptor cavity [47] (Fig. 3). Part of the requirement for this is the lack of bulky substituent groups. For example, although many of the compounds had substitutions at positions 5 and 11, those in which these substitutions were larger than a methyl group had considerably lower affinity. Secondly, the compounds should be planar and polycyclic heteroaromatic. As previously suggested [47], these two properties appear to be required for the best binding activity of agonists or antagonists. Finally, an antagonist should possess an electron-rich heteroatom e.g. ring nitrogen, near or along an otherwise relatively unsubstituted X-axis terminus of the van der Waals cavity (Fig. 3). The ring nitrogen especially was a consistent feature of compounds **1, 7, 19,** and **27,** as well as certain phenanthrolines [30].

Flavones

Of the compounds tested that showed antagonist activity, few had as high affinity for the receptor as ANF (calculated K_i of 7.7 nM; Table 1). Based on our initial hypothesis proposed above, four ANF derivatives containing amino or nitro substitutions at an X-axis terminal position (compounds **33–36;** Table 2) were synthesized and assayed for their relative binding affinities and for the stimulation and/or inhibition of DRE-binding activity as described for the ellipticine derivatives. None of these flavones had a relative affinity for the AhR as high as ANF (Table 2). However, an analysis of the combined AhR and DRE binding data for these compounds provided information that was consistent with the hypothesis for chemical features that enhance antagonist activity.

The amino substitution at the 4′-position (compound **36**) caused a significant loss of AhR affinity. In contrast, a nitro group at this same position (compound **34**) had much less of an effect on the relative affinity as compared to ANF. Compound **33,** with a nitro substitution at the 3′ position, had an approximately 3- to 4-fold less affinity. However, the IC_{50} value for the inhibition of TCDD-elicited DRE binding for compound **33** was close to the IC_{50} value for competition of TCDD-AhR binding (ratio of 0.9, Table 2), and there was a lack of significant DRE binding elicited by this compound alone (Table 2). The IC_{50}-DRE bind-

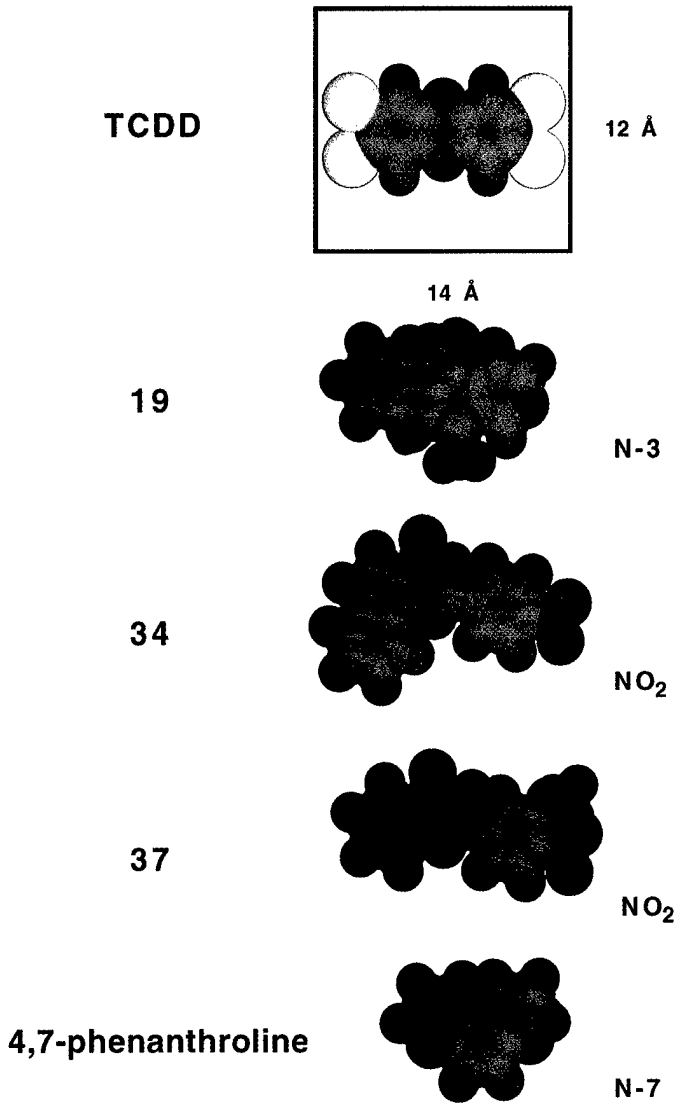


FIG. 3. Space-filling models for AhR-binding molecules. The predicted [45] van der Waals dimensions along the X and Y axes, 14 and 12 Å, respectively, are shown. The locations of the electron-rich groups, ring nitrogen or nitro, are given for compounds 19, 34, 37, and 4,7-phenanthroline.

ing/IC_{50}-AhR binding ratio was also near 1 for compound **34.** In comparison, this ratio for ANF was 1.7 (Table 1). Thus, although compounds **33** and **34** had a lower affinity for the AhR than ANF, their relative capacity for antagonism (i.e. relative to a comparable amount of AhR bound) appeared to be greater.

Based on the above data and in part on data published [48] while this work was being completed, compounds **37–40** were synthesized. Compound **37,** with an electron-withdrawing group at the 4′ position, proved to be a very potent antagonist as determined by both the relative ability to compete with [^{3}H]TCDD for binding to the AhR and the inhibition of TCDD-stimulated DRE binding (Table 2). The presence of the methoxy group at the 3′ position, as in compound **37,** and the ring carbons between positions 7 and 8, as in compounds **34** and **39,** increased the affinity of receptor binding. Compound **38,** which had neither of these substitutions, had considerably lower affinity, whereas compound **39,** which had both of these substitutions, had an affinity higher than compounds **34** and **37.** However, these data also suggest that the ring carbons between positions 7 and 8 contribute to agonist activity, since greater DRE-binding was observed with compound **39** as compared with compound **37.** Whatever effect the methoxy group at the 3′ position had appeared to be highly dependent on the presence of the nitro group at the 4′ position. An amino substitution at this position (compound **40**) significantly decreased both the binding affinity and the antagonist activity (as indicated by the increased IC_{50}-DRE binding/IC_{50}-AhR binding ratio).

Examination of Agonist/Antagonist Activity of Ellipticines and Flavones in Intact Cells

The ability of the more potent of the above chemicals to inhibit TCDD-elicited transformation of the AhR to an active form within intact cells was examined. p2Dluc contains 2 DREs that confer AhR-dependent TCDD-inducible control upon a minimal promoter linked to the luciferase gene. Hepa 1c1c7 cells transfected with this plasmid were found to be highly responsive to TCDD (Fig. 4 legend), whereas cells transfected with the plasmid containing the

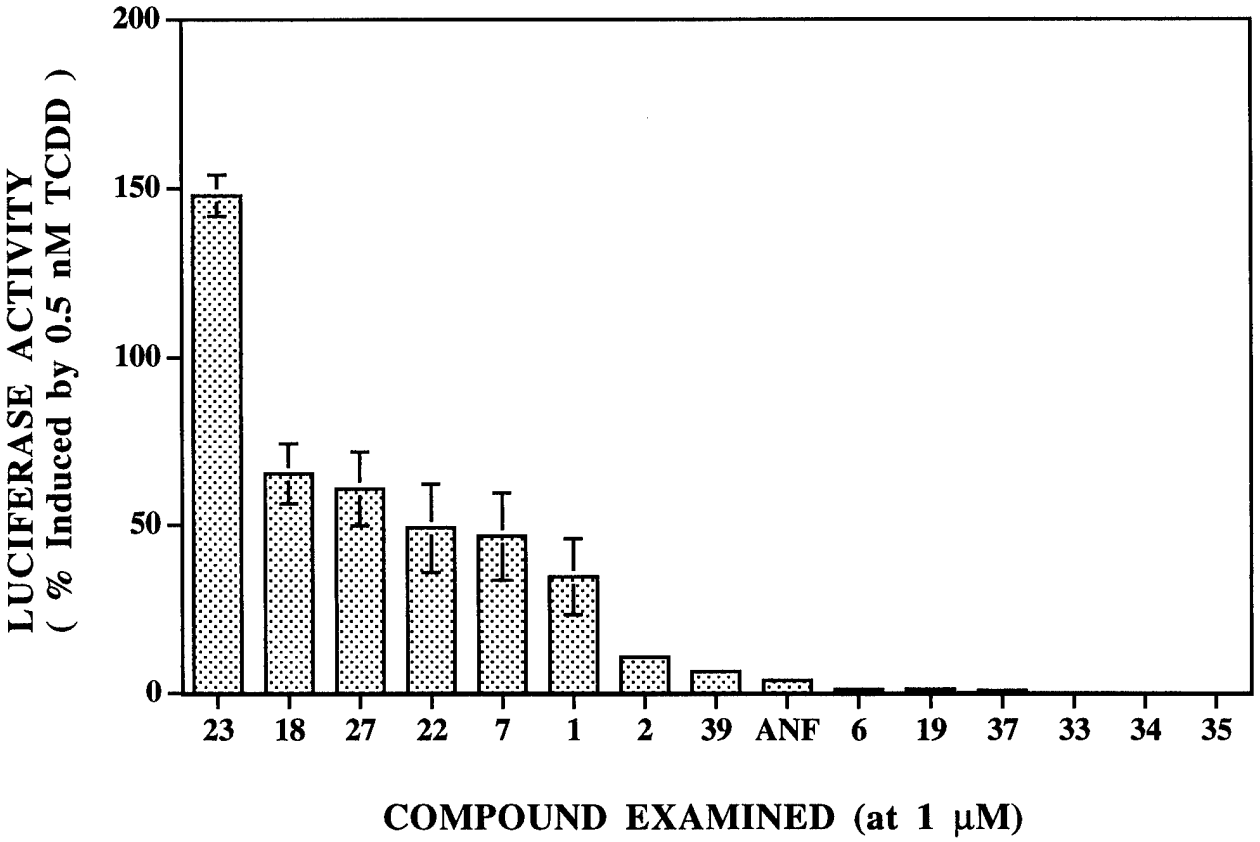


FIG. 4. Agonist activity of various compounds in Hepa 1 cells containing a luciferase reporter construct. Cells were exposed to 1 μM concentrations of the individual compounds or 0.5 nM TCDD at 8 hr prior to harvesting and determination of luciferase activity. Results are reported as the percent activity observed for TCDD in a particular experiment ± SD. For 4 separate experiments, the induction of luciferase by TCDD ranged from 20- to 38-fold over background (cells + *p*-dioxane), and within each experiment the variation of TCDD-induced levels from the mean was always <21%. The background ranged from 20 to 66 light units, and the absolute value observed following TCDD treatment averaged 2761 light units.

construct without the 2 DREs (pTATAluc) showed no alteration in luciferase activity following TCDD treatment (not shown). It should be noted that we chose not to examine the effect of these compounds on the induction of CYP1A1 activity since some of these may be competitive inhibitors of CYP1A1-dependent enzyme activity [48, 49].

As indicated by the *in vitro* data (Table 1), compound **23** was expected to have good agonist activity. The data obtained using the luciferase assay system were in agreement (Fig. 4). Of the ellipticine derivatives examined, **6** and **19** shows no significant induction of luciferase at a concentration of 1 μM, whereas **1, 2, 7, 18, 22,** and **27** elicited significant induction. The rank order for the ability of these compounds to induce luciferase in Hepa1c1c7 cells at a concentration of 1 μM was essentially the same as that noted for their ability to activate the AhR to a DRE-binding state under *in vitro* conditions using rat hepatic cytosol (see Table 1). The flavone derivatives **33, 34, 35,** and **37** showed no significant induction of luciferase, whereas ANF and compound **39** demonstrated a small, but detectable, level of induction (Fig. 4).

Compounds **1, 2, 6,** and **19,** were roughly equipotent in their ability to block TCDD-induced luciferase, with approximate IC_{50} values of 37–78 nM (Table 3). Two of the synthesized flavone derivatives (compounds **33** and **35**) with nitro and amino substitutions at the 3′ position were less potent in their ability to antagonize induction. Compound **34** demonstrated good antagonist activity, with an IC_{50} value near to that observed with compound **37.**

TABLE 3. Effect of various compounds on TCDD-induced luciferase activity in Hepa cells

Compound	Approximate IC_{50} values (nM)*
1	78 ± 35
2	48 ± 22
6	37 ± 16
19	52 ± 20
33	129 ± 65
34	22 ± 4
35	192 ± 32
37	13 ± 1
39	67 ± 34
ANF	80 ± 30

* Hepa cells were transfected with p2Dluc as described in Materials and Methods and exposed to 0.5 nM TCDD with and without .001 to 1.0 μM of test compounds. Cells were incubated for 8 hr and the activity of luciferase was determined as described in Materials and Methods. IC_{50} Values are the mean ± SEM of triplicate determinations.

DISCUSSION

A number of compounds that bind to the AhR and have weak agonist activity have been shown to antagonize some biochemical and biological responses induced by TCDD. These compounds include several polychlorinated biphenyl congeners [23], 1-amino-2,7,8-trichlorodibenzo-*p*-dioxin [24], 6-methyl-1,3,8-trichlorodibenzofuran [25], ANF [26, 50] and other flavone derivatives [48], 1,7- and 4,7-phenanthroline [30], as well as certain benzocoumarins [49] and ellipticines [28, 29, 50, 51]. Other than conforming to the structural requirements necessary for AhR binding as originally predicted by Poland and Knutson [52], revised by Gillner *et al.* [53], and recently refined by Waller and McKinney [47], there are no obvious structural similarities among these compounds that would suggest requirements for antagonist activity. This may be related, in part, to different endpoints examined as well as the potential of these compounds to elicit antagonist activity by different mechanisms. Nevertheless, a determination of any consistent structural features necessary for antagonist activity would be valuable in the identification and/or synthesis of potent antagonists that could be used as probes to define the ligand-dependent changes that regulate AhR activity. This would further help to define the relationships among AhR actions, TCDD-induced altered gene expression and elicited biological and/or toxicological responses, as well as determine the normal function of the AhR. In an attempt to define such features, a number of ellipticine derivatives and structurally-related compounds were examined in this study. This class of compounds was selected based on the ability of certain of them to bind to the AhR [28], inhibit its transformation to a DNA-binding state [29], and inhibit benzo[*a*]pyrene hydroxylase activity [28, 51], a TCDD-inducible response.

Of the ellipticine derivatives examined, only a few demonstrated high affinity for the AhR. Although all of these bound with less affinity than TCDD, the structural requirements for binding agreed with those constraints previously described [47, 53] in terms of being planar aromatic compounds with approximate van der Waals dimensions of 14 × 12 × 5 Å, and with few bulky substituent groups. It is of interest that the K_i values of certain ellipticines for inhibition of TCDD binding (Table 1) were in some cases within the same order of magnitude or lower than those concentrations found to be effective in the stimulation of topoisomerase II-mediated DNA cleavage [54]. However, the presence of the 9-hydroxy group appears to be crucial in the stabilization of the topoisomerase II-DNA complex [54], and thus the structure-activity relationships for AhR binding appear to be very different than for topoisomerase II-mediated DNA cleavage. Nevertheless, it is possible that the biological activity of the AhR may be modulated under conditions used for the chemotherapeutic effectiveness of ellipticine derivatives [55].

The potential agonist activity of these compounds was determined by examining their relative ability to activate the AhR to a DRE-binding form. The rutaecarpines, along with compounds **18, 22,** and **23,** showed the highest AhR binding affinities and greatest ability to stimulate AhR-DRE-binding activity. Quantitatively, however, many other compounds induced much less DRE-binding than what might be predicted from the K_i values for AhR binding assuming full agonist activity of the bound receptor. A striking example of this is compound **19.** Although the K_i for the inhibition of TCDD binding is approximately 12 nM, a concentration of 1 μM elicited a barely detectable amount of DRE-binding (Table 1 and Fig. 1). For a number of other compounds e.g. **1, 7,** and **27,** this property appeared to be consistent with the presence of an electron-rich ring nitrogen along a lateral position of the designated van der Waals dimensions. Thus, the presence of this electron-rich center substantially decreased the efficacy of the particular compound to activate the AhR to a DRE-binding form.*

When the data for AhR binding and stimulation of DRE binding are considered together with the ability of these chemicals to inhibit TCDD-elicited DRE binding, only compounds **1, 7, 19,** and **27** had characteristics consistent with properties of good antagonists. Again, compounds with the most potent antagonist activity appeared to fit the predicted van der Waals dimensions, and had an electron-rich ring nitrogen at a lateral position. A comparison of compounds **19** and **22,** which differed only in the presence of the ring nitrogen at position 3 (Table 1), offered the best illustration of this. Despite the approximately 4-fold higher AhR affinity of compound **22** as compared with compound **19,** compound **19** has an approximately 2-fold greater ability to inhibit TCDD-elicited DRE-binding. A possible exception to this pattern was compound **18** which appeared to have reasonably good ability to induce AhR-DRE binding. In this case, however, the presence of the amino substitution at position 1 may cause some delocalization of electrons associated with the adjacent ring nitrogen, or may sterically hinder the nitrogen from interacting with a particular site in the ligand-binding pocket of the AhR.

In this study we examined the ability of these chemicals to stimulate/inhibit DRE binding as a means to measure their potential AhR agonist/antagonist activity. However, it is inappropriate to assume that DRE binding under cell-free conditions accurately reflects the potential ability of any AhR ligands to modulate gene expression in whole cells and/or tissues. A variety of factors, such as cell uptake and metabolism, and regulatory influences on the cell- and temporal-specific transcriptional activity of a particular gene are crucial in determining the final response *in vivo*. Also, the presence of a DRE-binding complex does not assure that the complex is transcriptionally active. To ad-

* The notion of decreased efficacy of weak agonists or partial antagonists is consistent with the receptor theory as described by Stephenson [56]. Nevertheless, this is a principle that is largely ignored or not thoroughly considered when evaluating the potential ability of mixtures of compounds to elicit AhR-mediated biological and/or toxicological responses.

dress these issues, we also examined for the ability of these compounds to induce the luciferase gene under control of a DRE-containing enhancer following the transfection of a plasmid containing these elements into Hepa cells known to be responsive to TCDD. These data indicated a good agreement between the ability of the ellipticine derivatives to activate the AhR complex to a DRE-binding form *in vitro* compared to the DRE-driven induction of luciferase activity in whole cells (compare Table 1 and Fig. 4).

Compounds **33–36** synthesized in this study had lower affinity than the parent compound (ANF). Nevertheless, the data obtained from the analysis of these were consistent with the prediction of an electron-rich center (in this case, electron-withdrawing oxygens of a nitro group) at a lateral position of the molecule being important for antagonist activity. Both **33** and **34,** with nitro substitutions at positions 3′ and 4′, respectively, appeared to have good efficacy as antagonists as demonstrated by the lack or low level of AhR activation to a DRE binding form *in vitro*, the inability to stimulate (at least up to 1 μM) luciferase activity in the transfected Hepa 1c1c7 cells, and the relative ability to block TCDD-elicited luciferase activity. Of these, **34** exhibited the greatest potency.

Data from these derivatives were also useful in interpreting a recent publication describing the ability of 3′-methoxy-4′-nitroflavone (compound **37**) to antagonize TCDD-elicited responses in MCF-7 human breast cells [48]. Using both rat AhR *in vitro* and murine AhR in the transfected cell model, this compound, in our hands, also proved to be a potent and possibly pure AhR antagonist. Furthermore, the structure of this compound was consistent with our hypothesis regarding structural requirements for antagonist activity. In addition, the data obtained with this compound were qualitatively similar to those seen with compounds **34** and **39,** both of which also had a nitro substitution at the 4′ position. It was of particular interest that the presence of the methoxy group at the 3′ position greatly contributed to the high affinity for the receptor. A good explanation for this may reside in the ability of the electrons from the methoxy group oxygen to be delocalized through resonance, thus permitting a greater increase in the electron density of the nitro group oxygens. The dependence of the effect of the methoxy group on the nitro group, as compared with the less electron-rich amino group (in compound **40**), at the 4′ position is also consistent with this possibility. Work by Lu *et al.* [48] also demonstrates the importance of the positioning of the methoxy group at the 3′ position, since this substitution at the 4′ position with nitro- or amino-substitutions at the 3′ position significantly decreased the potency of ligands.

It will be of interest to examine if our determined chemical characteristics for antagonist activity are consistent for AhR molecules from a variety of species. There may be species-specific differences in the three-dimensional structure of the receptor at the ligand-binding site or other domains that could influence agonist/antagonist activity. Although we and others have reported ANF to be an effective antagonist of rat AhR activity [27, 40], recent studies indicate a lack of such antagonism of the guinea pig AhR complex [47]. The cells used in the present study were derived from a mouse hepatoma, whereas our source of receptor for the *in vitro* studies was rat liver. As indicated above, this appeared not to influence to a large degree that qualitative nature of the agonist responses observed. However, some quantitative disparities might be due to species-specific differences in AhR structure and regulation. For example, Fernandez *et al.* [28] noted approximate 8- to 20-fold differences in the IC_{50} values for both ellipticine (compound **1**) and 9-hydroxyellipticine (compound **4**) to inhibit TCDD binding to AhR in rat and mouse liver, with the rat AhR having higher affinities. This may, in part, explain the relatively good antagonist activity of compound **1** observed *in vitro* with rat AhR (Table 1) as compared to the poorer antagonist activity obtained using mouse Hepa 1c1c7 cells (Fig. 4 and Table 3).

Certain ellipticine and flavone derivatives identified in this study and that by Lu *et al.* [48] represent two of the most potent groups of AhR antagonists identified to date. Compounds **6, 19, 34** and **37** showed no significant induction of DRE-driven luciferase activity in mouse hepatoma cells at concentrations up to 1 μM (Fig. 4), while inhibiting TCDD-induced luciferase at IC_{50} concentrations between 13 and 52 nM (Table 3). The antagonist activity of both groups appears to depend, at least in part, on the presence of an electron-rich center, i.e. ring nitrogen or a nitro group at a lateral position (Fig. 3). Likewise, the ability of 1,7- and 4,7-phenanthroline to inhibit TCDD-induced responses *in vitro* [30] and *in vivo* [58] may depend on the same structural characteristics (Fig. 3); although the smaller van der Waals dimensions of these likely are responsible for their lower affinity for the AhR. The presence of similar structural features among other compounds reported to have antagonist activity is less obvious. We did not detect any inhibition of AhR-TCDD binding to the DRE by 1-amino-3,7,8-trichlorodibenzo-*p*-dioxin *in vitro* up to 1 μM (data not shown). Either the mechanism of antagonism *in vivo* [24] is very different, or the compound is metabolized to a product that permits the formation of an electron-rich center. Similar considerations hold for several polychlorinated biphenyl congeners reported to antagonize some of the actions of TCDD [23].

The exact mechanism(s) by which these compounds act remains to be determined. The mechanism of antagonism for those compounds possessing an electron-rich region at a lateral position might be very different from other compounds having antagonist activity. We have shown that certain concentrations of ANF block activation of the AhR to a DRE-binding state [40]. The present data for the ellipticines and flavone derivatives are consistent with this general mechanism. We have also suggested [40] that ANF may "lock" the receptor in a conformation that does not readily dissociate from hsp90, thus preventing the formation of the heterodimer complex with Arnt protein. On the other hand, studies by Merchant *et al.* [59] suggest that

6-methyl-1,3,8-trichlorodibenzofuran may act by allowing the formation of a heterodimer complex that may bind to DNA but is less active at the transcriptional level.

In summary, data obtained from this study have been useful in identifying and further characterizing two groups of potent AhR antagonists. Furthermore, based on this information, we have determined that the antagonist activity of these compounds depends on the presence of an electron-rich center at an X-axis lateral position. These data will be useful in the further design and/or identification of potent and irreversible antagonists to be used as probes to understand the function of the AhR and its role in both the toxicity of the dioxins and related compounds. Further work is also required to determine the antagonist efficacy of these compounds in different species *in vitro* and *in vivo* and the mechanism(s) underlying this antagonist activity.

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