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

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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-

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§ *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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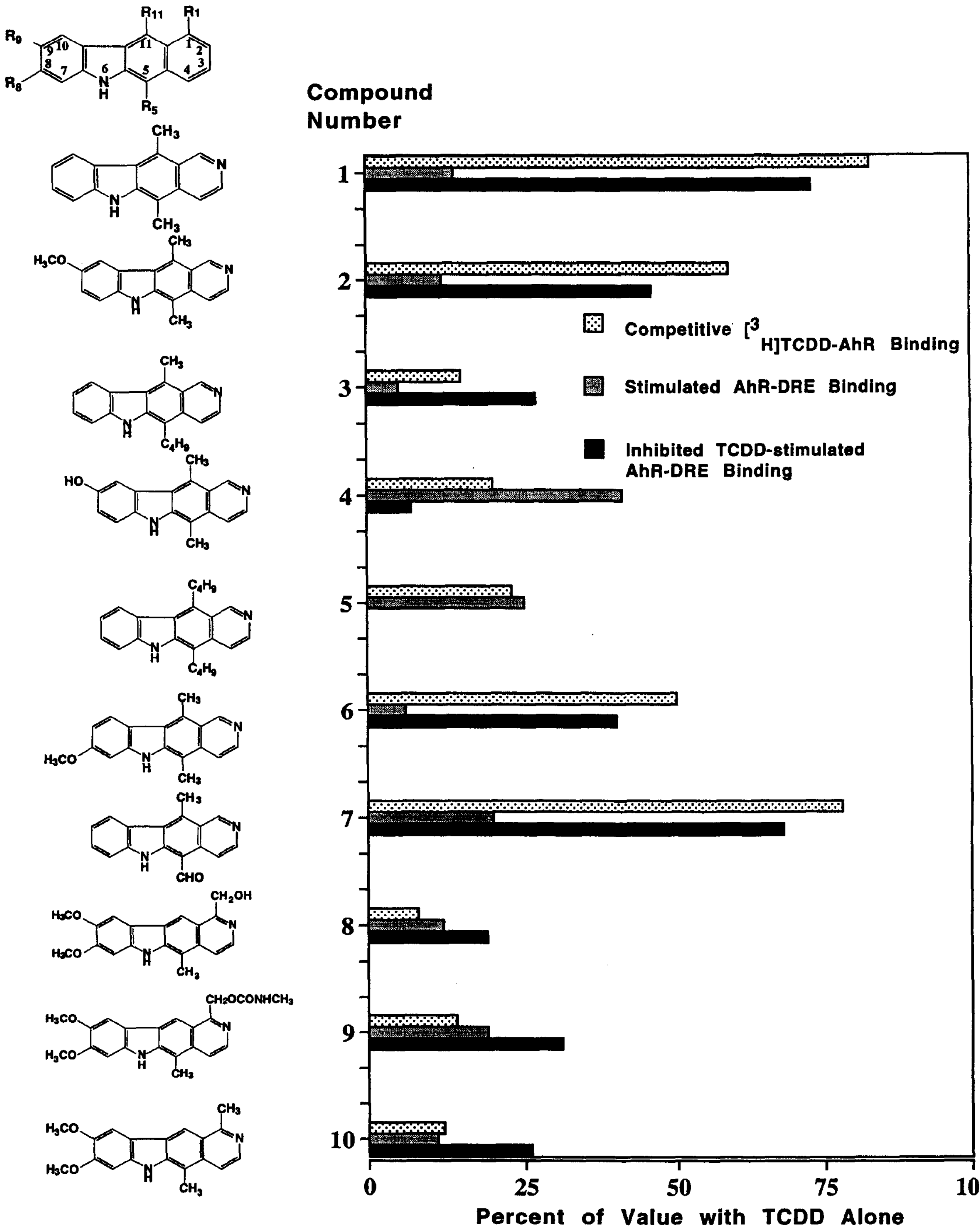

R11
R1
R9
R8
R5
1 2 3 4 5 6 7 8 9 10 11
N H
CH3
H3CO
HO
C4H9
CHO
CH2OH
CH2OCONHCH3
Compound Number
1
2
3
4
5
6
7
8
9
10
Competitive [3H]TCDD-AhR Binding
Stimulated AhR-DRE Binding
Inhibited TCDD-stimulated AhR-DRE Binding
0
25
50
75
100
Percent of Value with TCDD Alone

FIG. 1.

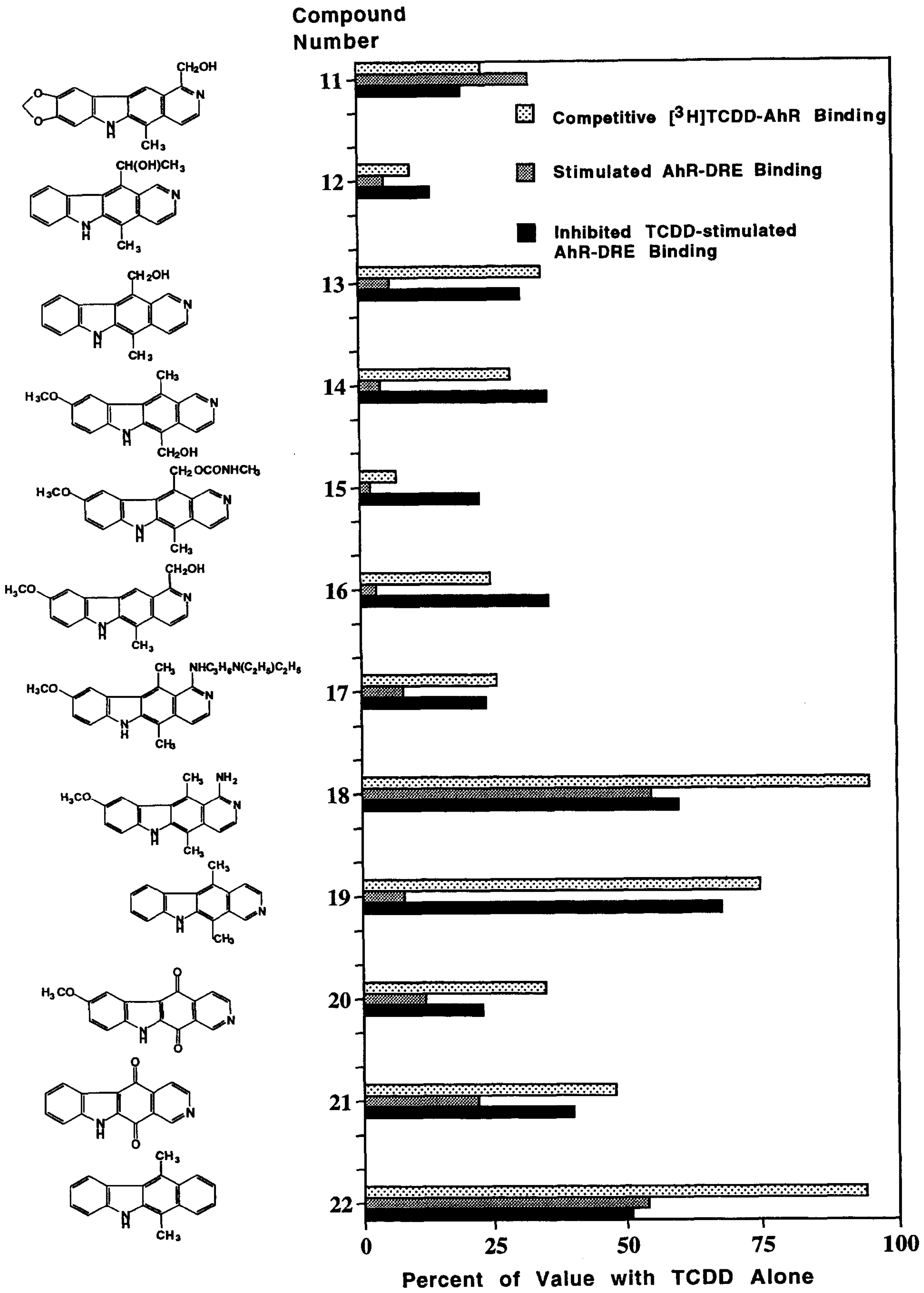


FIG. 1. Continued.

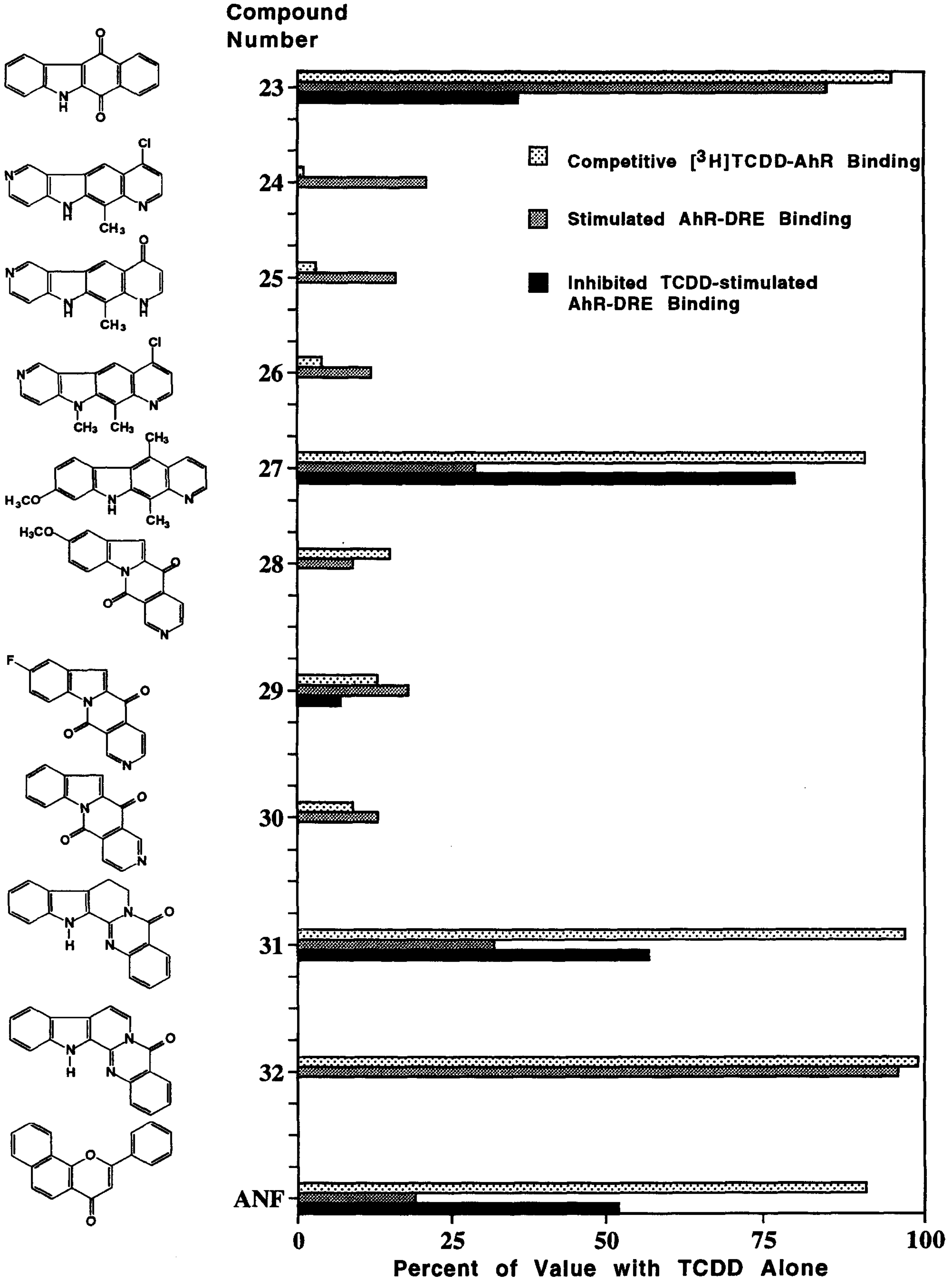


FIG. 1. Continued.

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

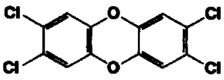
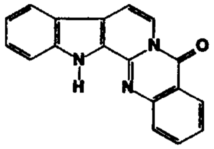
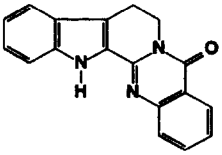
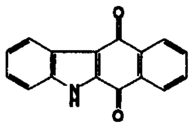
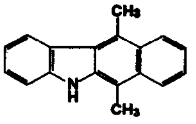
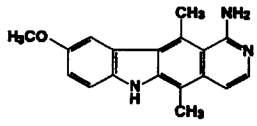
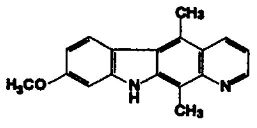
HEDG buffer consisted of 25 mM HEPES, 1.5 mM EDTA, 1 mM dithiothreitol, and 10% (v/v) glycerol, pH adjusted to 7.6 at room temperature.

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

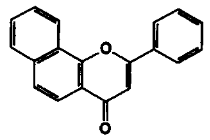
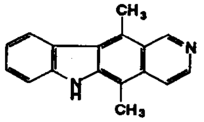
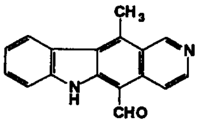
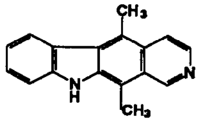
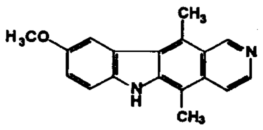
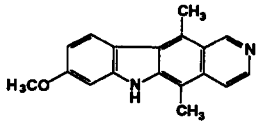
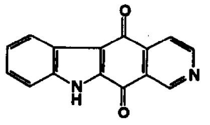
(continued)

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	$\frac{\text{IC}_{50}\ \text{(DRE) Binding}}{\text{IC}_{50}\text{(AhR Binding)}}$*
		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 $A = B - B/(1 \times (C/D)^e)$ 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].

Assessment of DRE Binding (EMSA)

The complementary oligodeoxynucleotides 5′-GATCCGGCTCTTCTCACGCAACTCCGAGCTCA-3′ (noncoding strand) and 5′-GATCTGAGCTCGGAGTTGCGTGAGAAGAGCCG-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

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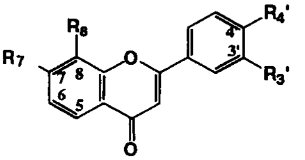
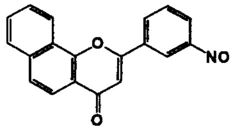
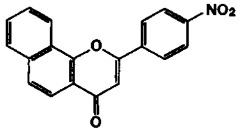
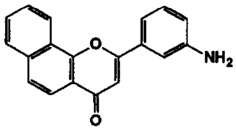
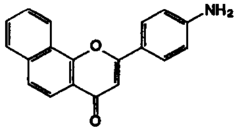
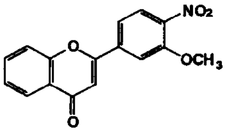
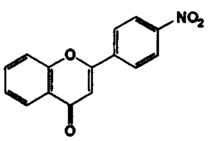
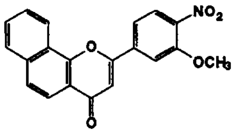
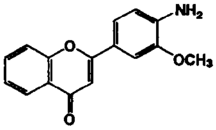
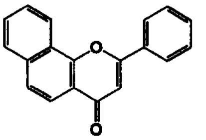
* At infinite dilution, the affinity of the AhR for TCDD is estimated to be 7–10 pM [43].

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**	**$\frac{\text{IC}_{50}\text{ (DRE) Binding}}{\text{IC}_{50}\text{(AhR Binding)*}}$**
		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

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* 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 *XhoI-HindIII* 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 1000g 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

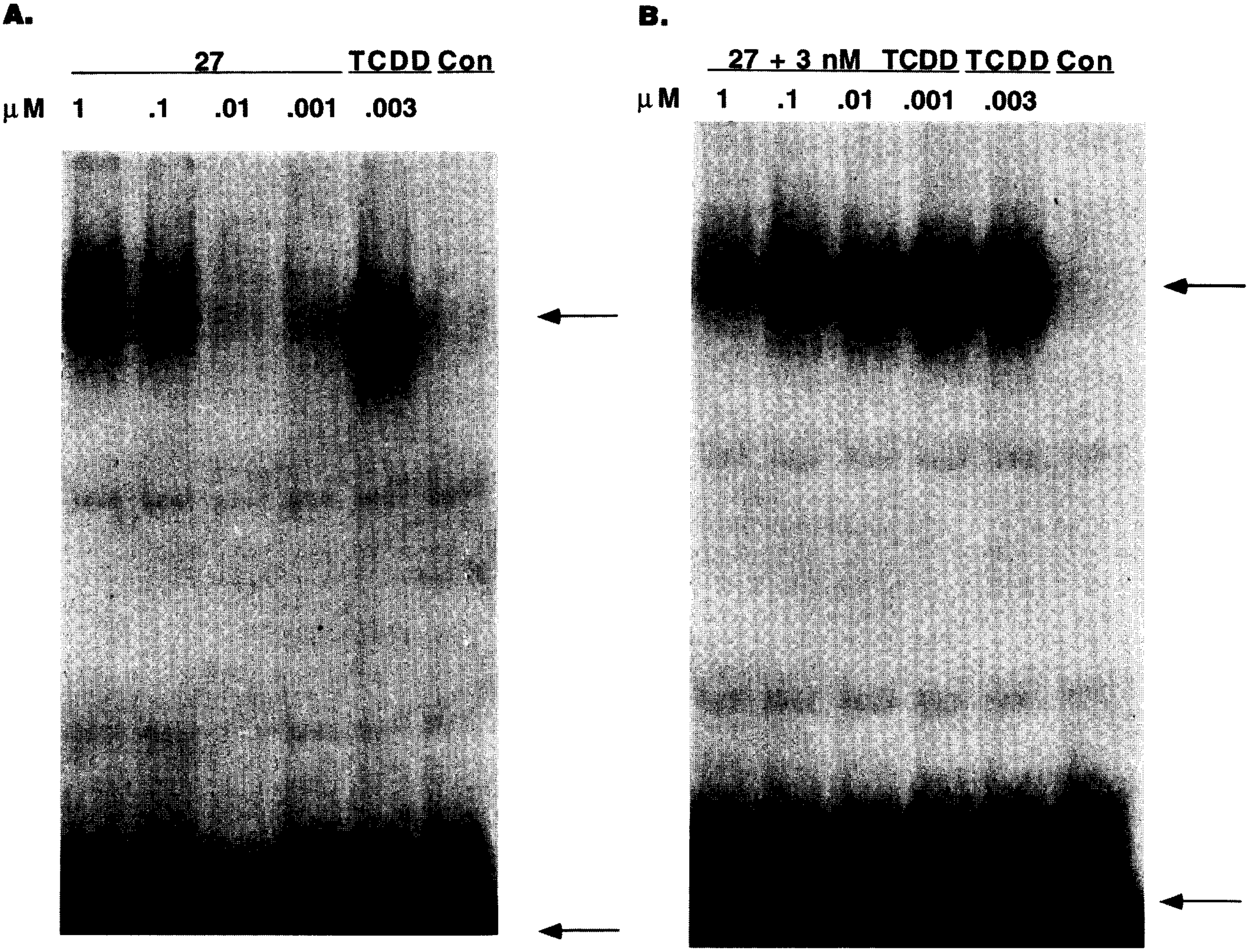

FIG. 2. EMSA of cytosol incubated with TCDD (3 nM) or compound 27 (A), or TCDD in the absence or presence of compound 27 (B). Rat hepatic cytosol was incubated with the indicated concentrations of ligand for 2 hr, 20° followed by EMSA as described in Materials and Methods. Top arrow indicates the AhR-bound ^{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-

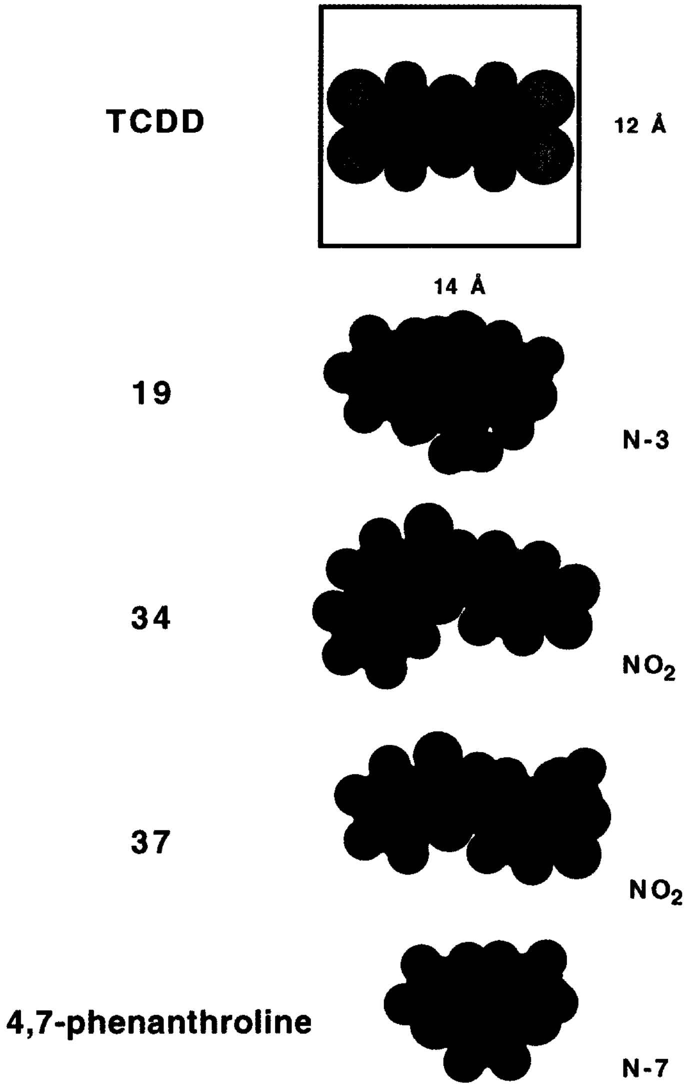

FIG. 3. Space-filling models for AhR-binding molecules. The predicted [45] van der Waals dimensions along the X and Y axes, 14 and 12 Å, respectively, are shown. The locations of the electron-rich groups, ring nitrogen or nitro, are given for compounds 19, 34, 37, and 4,7-phenanthroline.

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

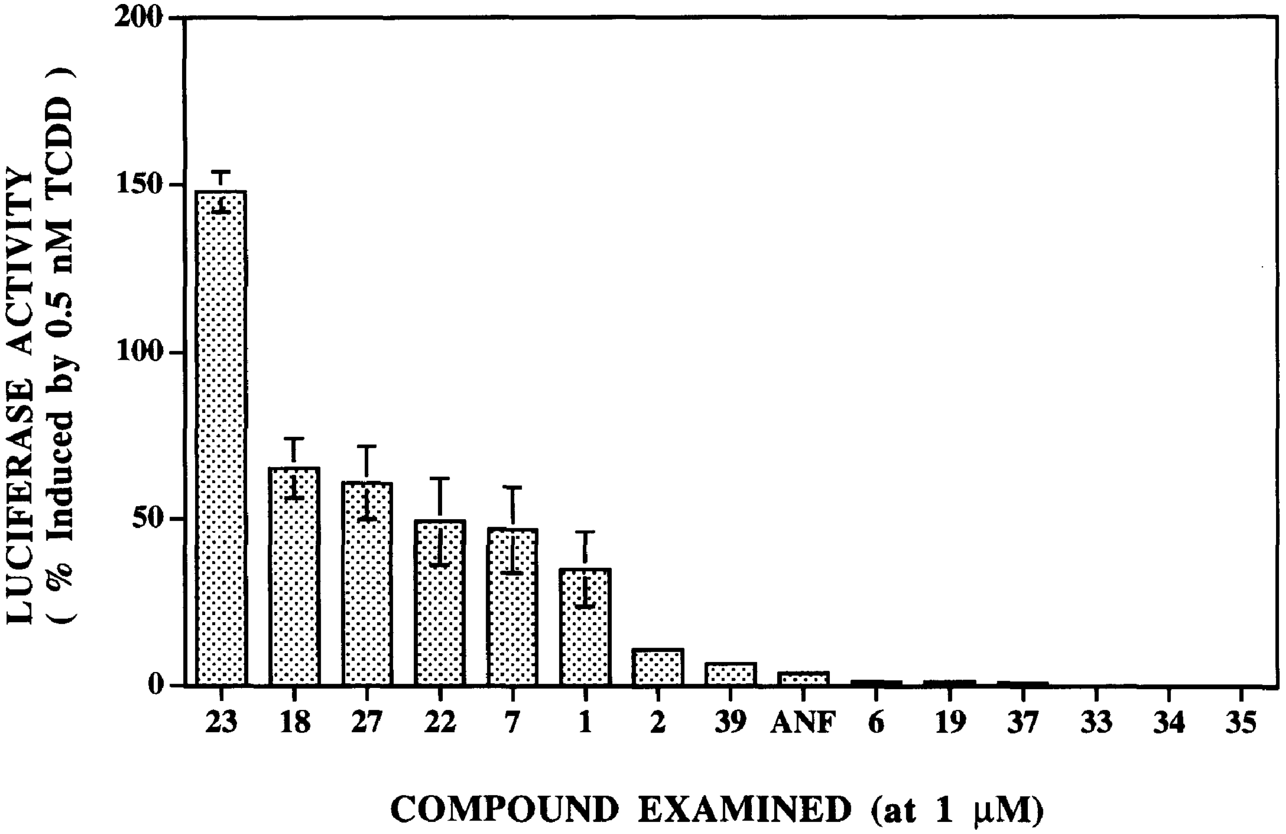

FIG. 4. Agonist activity of various compounds in Hepa 1 cells containing a luciferase reporter construct. Cells were exposed to 1 μM concentrations of the individual compounds or 0.5 nM TCDD at 8 hr prior to harvesting and determination of luciferase activity. Results are reported as the percent activity 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-

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* 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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