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Substituted Flavones as Aryl Hydrocarbon (Ah) Receptor Agonists and Antagonists

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ABSTRACT. The structure-dependent aryl hydrocarbon (Ah) receptor agonist and antagonist activities of the following substituted flavones were investigated: flavone, 4′-methoxy-, 4′-amino-, 4′-chloro-, 4′-bromo-, 4′-nitro-, 4′-chloro-3′-nitro-, 3′-amino-4′-hydroxy-, 3′,4′-dichloro-, and 4′-iodoflavone. The halogenated flavones exhibited competitive Ah receptor binding affinities (IC_{50} = 0.79 to 2.28 nM) that were comparable to that observed for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (1.78 nM). The compounds also induced transformation of the rat cytosolic Ah receptor and induced *CYP1A1* gene expression in MCF-7 human breast cancer cells. However, despite the high Ah receptor binding affinities for these responses, the halogenated flavones were >1000 times less active than TCDD for the other responses. Moreover, for other substituted flavones, there was no correlation between Ah receptor binding affinities and their activities as Ah receptor agonists. For example, 4′-aminoflavone induced CYP1A1 mRNA levels in MCF-7 cells but exhibited relatively low Ah receptor binding affinity (IC_{50} = 362 nM) and did not induce transformation of the rat cytosolic Ah receptor. All of the substituted flavones inhibited TCDD-induced transformation of the Ah receptor, and 4′-iodoflavone, an Ah receptor agonist at high concentrations (1–50 μM), inhibited the transformation at concentrations as low as 0.05 and 0.5 μM. Subsequent interaction studies with TCDD and 4′-iodoflavone confirmed that the latter compound inhibits induction of *CYP1A1* gene expression by TCDD in MCF-7 cells. The results obtained for the substituted flavones suggest that within this structural class of compounds, various substituent groups can affect markedly the activity of each individual congener as an Ah receptor agonist or antagonist. These substituent-dependent differences in activity may be related to ligand-induced conformational changes in the Ah receptor complex and/or support the proposed existence of more than one form of the Ah receptor. BIOCHEM PHARMACOL 51;8:1077–1087, 1996.

KEY WORDS. flavonoids; Ah receptor binding; *CYP1A1* induction/inhibition

TCDD‖ is an industrial and combustion by-product that has been widely identified as an environmental contaminant. TCDD elicits a diverse spectrum of biochemical and toxic responses and is used as a prototype for investigating the toxicology and mechanism of action of structurally related halogenated aromatic compounds [1–4]. Poland and coworkers [5] first demonstrated that [^{3}H]TCDD bound saturably and with high affinity to an hepatic cytosolic protein from C57BL/6 mice, and this protein, named the Ah receptor, has since been widely identified in animal and human tissues [6, 7]. The results of photoaffinity labeling studies indicated that there was considerable intraspecies variability in the apparent molecular weight of the Ah receptor [8], and this is consistent with results of experiments that have cloned the Ah receptor from various species [9–13]. The proposed mechanism of action of TCDD and related compounds was initially derived from studies on induction of *CYP1A1* gene expression by TCDD, 3-methylcholanthrene, and related compounds [3]. The unbound cytosolic Ah receptor is associated with heat shock protein 90; after binding with TCDD, the 9S (270- to 300-kDa) cytosolic Ah receptor complex undergoes a transformation step to form a 5- to 7S (180- to 210-kDa) heterodimeric complex that rapidly accumulates in the nuclear fraction of target cells [14, 15]. The nuclear Ah receptor complex acts as a ligand-induced transcription factor that interacts with 5′-dioxin or xenobiotic response elements (DREs, XREs), and this can result in transactivation of the *CYP1A1* gene [16, 17]. The nuclear heterodimeric complex consists of the Ah receptor and Ah receptor nuclear translocator (Arnt) protein [18], and there is strong evidence that formation of this

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‖ *Abbreviations:* Ah, aryl hydrocarbon; αNF, αnaphthoflavone; DRE, dioxin-responsive element; EROD, ethoxyresorufin *O*-deethylase; HAP, hydroxylapatite; MCDF, 6-methyl-1,3,8-trichlorodibenzofuran; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; and TCDF, 2,3,7,8-tetrachlorodibenzofuran.

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unique ligand-induced dimeric protein complex plays a major role in modulating expression of several genes [19].

In vitro studies with TCDD and related halogenated aromatics show that there is a rank order correlation between their Ah receptor binding affinities and biochemical/toxic potencies [2, 20, 21]. Moreover, for TCDD and related halogenated aromatic compounds, there is also a correlation between their Ah receptor binding affinities and their activity to induce transformation (i.e. 9–10S to 5–7S) of the rat hepatic cytosolic receptor [22]. The correlations observed for halogenated aromatics have not been investigated extensively within other structural classes of compounds that bind to the Ah receptor. For example, 3-methylcholanthrene binds with high affinity to the Ah receptor but is a relatively weak Ah receptor agonist for several responses [23]. This disparity between binding affinity versus potency as an Ah receptor agonist can be explained, only in part, by the rapid metabolism of 3-methylcholanthrene [24, 25]. However, in ligand-induced cytosolic Ah receptor transformation experiments, 3-methylcholanthrene is significantly less active than TCDD, despite their comparable Ah receptor binding affinities [24].

3′-Methoxy-4′-nitroflavone also binds with high affinity to the rat cytosolic Ah receptor (IC_{50} = 2.27 nM) but does not induce significantly CYP1A1 mRNA levels in MCF-7 human breast cancer cells nor does it induce *in vitro* transformation of the rat hepatic cytosolic Ah receptor [26]. 3′-Methoxy-4′-nitroflavone is an Ah receptor antagonist that inhibited formation of the nuclear Ah receptor complex and induction of *CYP1A1* gene expression by TCDD in MCF-7 cells. This study further investigates the structure-dependent activities of 4′- and 3′,4′-substituted flavones as Ah receptor agonists/antagonists in MCF-7 cells. The haloflavones bound with high affinity to the cytosolic Ah receptor (0.79 to 2.28 nM) but were >1000-fold less active than TCDD as inducers of *CYP1A1* gene expression. Moreover, 4′-iodoflavone was a partial antagonist. There were no apparent structure–activity relationships for the other substituted flavones.

MATERIALS AND METHODS

Chemicals and Biochemicals

The 3′,4′-substituted flavones were synthesized and were >98% pure as determined by spectroscopic measurements and HPLC as described previously [27]. TCDD, [^{3}H]TCDD, ethoxyresorufin, and TCDF were synthesized in this laboratory to >98% purity. MCF-7 human breast cancer cells were obtained originally from the American Type Culture Collection (ATCC, Rockville, MD). All other chemicals and biochemicals were of the highest purity available from commercial sources.

Preparation of Rat Hepatic Cytosol

Liver cytosol from Long-Evans rats was prepared as previously described [26]. Livers were perfused *in situ* with ice-cold HEGD buffer [25 mM HEPES, 1.5 mM EDTA, 1.0 mM dithiothreitol, 10% glycerol (v/v), pH 7.8], homogenized in HEGD (5 mL/g tissue), and centrifuged at 10,000 *g* for 20 min (2°). The resulting supernatant was centrifuged at 105,000 *g* for 1 hr (2°), and the supernatant was stored at −80°. Protein concentrations were determined by the method of Bradford [28].

Cytosolic Receptor Binding Assay

The IC_{50} = and K_i values for competitive receptor binding affinities were determined using rat hepatic cytosol (2 mg protein/mL) and the hydroxylapatite assay procedure essentially as described [29]. Each sample was incubated with [^{3}H]TCDD and the desired competitor at 20° for 2 hr. After incubation, 200 μL of incubate was added to fresh tubes containing HAP slurry (35 mL HAP/100 mL HEGD buffer). Tubes were allowed to incubate for 30 min and centrifuged at 100 *g* for 5 min (4°). The resulting pellet was washed three times with ice-cold HEGD containing 1% Triton X-100. The final pellet was then rinsed with 1 mL ethanol, and the radioactivity was determined by liquid scintillation counting. Non-specific binding was obtained by incubating [^{3}H]TCDD with a 200-fold excess of TCDF. Different concentrations of the ligands were used to determine displacement curves; the IC_{50} = values were defined as the concentrations required to displace 50% of [^{3}H]TCDD and were determined from a log-logit plot of the percentage of [^{3}H]TCDD bound versus log concentrations of the ligands. The results are expressed as means ± SEM for at least 4 separate determinations. K_i values were determined by the method of Cheng and Prusoff [30], as described previously [26].

Ligand-Induced Transformation Assay

Polyacrylamide gel electrophoresis purified complementary strands of the synthetic oligonucleotide, containing a 26-mer consensus DRE sequence (5′-GATCTGGCTCTTCT-CACGCAACTCCG-3′) that corresponds to the DNA sequence from -1997 to -1978 of the mouse *CYP1A1* gene [16], was obtained from Genosys Biotechnologies, Inc. (The Woodlands, TX). The oligonucleotide was labeled at the 5′-end using T4-polynucleotide kinase and [γ-^{32}P]ATP. DNA binding was measured using a gel mobility shift assay [16]. Rat hepatic cytosol (16 mg/mL protein) was incubated for 2 hr at 20° with 5 nM TCDD, 50 μM flavone or 5 nM TCDD plus different concentrations of the flavones; the final concentration of DMSO was 0.2% (v/v). Cytosol (80 μg) was incubated in HEGDK (HEDG + 0.4 M potassium chloride) with 400 ng of poly[d(I-C)] in HEGD buffer and incubated for 15 min at 20°. Following the addition of a ^{32}P-labeled DRE oligonucleotide (0.2 to 1.0 ng; 100,000 cpm), the mixture was incubated for an additional 15 min at 20°. Excess unlabeled DRE (100-fold) was added 5 min prior to addition of ^{32}P-labeled DRE to compete for the specific DNA–protein binding. Protein–DNA complexes

were resolved on a 5–6% polyacrylamide gel (acrylamide: bisacrylamide; 30:0.8) and electrophoresed at 120 V for approximately 3 hr in 0.9 M Tris borate and 2 mM EDTA, pH 8.0. Gels were dried, and protein–DNA complexes were detected using a Betagen Betascope 603 blot analyzer and visualized by autoradiography. The amount of ^{32}P-labeled DRE bound in the ligand-inducible complex was determined by measuring the radioactivity of the specifically bound retarded band and subtracting the amount of radioactivity present in the same position in a non-ligand-treated lane. The difference in radioactivity between these samples represents the ligand-inducible specific binding of the Ah receptor complex to the [^{32}P]DRE. The levels of DNA-binding activity for the various treatment groups were quantified as a percentage of maximal response observed for the TCDD-treated cytosol. The IC_{50} values were obtained from a log-logit plot of the percentage of maximal response obtained for each ligand versus ligand concentration.

Inhibition of Microsomal EROD Activity by Substituted Flavones

Male Long-Evans rats were treated with TCDD (5 μg/kg), and induced rat hepatic microsomes were isolated as described previously [31]. The substituted flavones (1 μM) were incubated with rat liver microsomes (25 μg protein/incubation) for 10 min as described previously [26], and EROD activity was determined spectrofluorimetrically [32].

Cell Growth, Induction, and Inhibition of EROD Activity

MCF-7 cells were grown in medium containing DME/F12 with 2.2 mg/mL sodium bicarbonate, 5% fetal bovine serum, and 10 nM antibiotic-antimycotic solution (Sigma Chemical Co., St. Louis, MO). For determination of EROD activities, the cells were first seeded into 60-mm petri dishes; 1 nM TCDD, different concentrations of the substituted flavones, and TCDD plus the flavones were dissolved in DMSO (0.1%) and added to the culture dishes when the cells reached 70% confluence. Cells were harvested 24 hr after chemical treatment, and EROD activity was determined fluorimetrically [32].

Northern Analysis for CYP1A1 mRNA Levels

The murine CYP1A1 cDNA probe was obtained from ATCC. The plasmid pGMB1.1 containing the mouse β-tubulin cDNA was a gift from Dr. Don Cleveland (Johns Hopkins University). Digestion of the plasmid yielded a 1.3 kb fragment that was used to detect β-tubulin mRNA. RNA from the cells treated with TCDD or substituted flavones for 24 hr was isolated, electrophoresed, transferred to a nylon membrane, and probed by autoradiography or using a Betagen Betascope 603 imaging system as described previously [33, 34].

Statistical Analysis

The statistical differences between different treatment groups were determined by ANOVA and Student's *t*-test, and the levels of probability are noted ($P < 0.05$ or $P < 0.01$). The data are expressed as means ± SEM or deviations SD for at least 3 determinations for each experimental point.

RESULTS

A series of ten 4′- and 3′,4′-substituted flavones were synthesized previously [27] and used in the present study. The results in Table 1 summarize the IC_{50} and K_i values obtained for the concentration-dependent displacement of [^{3}H]-TCDD from the rat cytosolic Ah receptor. The IC_{50} values for 4′-chloro-, 4′-bromo-, 4′-iodo-, and 3′,4′-dichloroflavone varied from 0.79 to 2.28 nM and were comparable to the IC_{50} value obtained for unlabeled TCDD (1.78 nM). The most active of the four compounds was 4′-iodoflavone (IC_{50} = 0.79 nM); all the remaining substituted flavones exhibited >100 times lower competitive binding affinities for the Ah receptor (IC_{50} values from 99 to 4697 nM). The K_i values exhibited comparable structure–activity relationships.

The effects of structure on ligand-induced transformation of the rat hepatic cytosolic Ah receptor were determined using gel mobility shift assays followed by quantitating the transformed Ah receptor-[^{32}P]DRE complex using a Betagen Betascope 603 blot analyzer. The effects of a 50 μM concentration of the substituted flavones were compared with the intensity of the retarded band observed with 5 nM TCDD. The intensity of the retarded band observed for 50

TABLE 1. Competitive binding of substituted flavones to rat hepatic cytosol Ah receptor and their activity to induce transformation of the rat cytosolic Ah receptor

Compound				DRE binding†
R (3′)	R (4′)	IC_{50}* (nM)	K_i* (nM)	(%)
H	H	216 ± 110	138	31 ± 6.0
H	OCH_3	99 ± 45	63	28 ± 7.7
H	NH_2	362 ± 128	232	ND‡
H	Cl	1.40 ± 0.4	0.92	351 ± 67
H	Br	1.50 ± 0.7	0.96	99 ± 10
H	NO_2	4697 ± 1991	2453	ND
NO_2	Cl	1935 ± 1139	1240	2.4 ± 1.2
NH_2	OH	738 ± 371	506	ND
Cl	Cl	2.28 ± 0.5	1.47	75 ± 12
H	I	0.79 ± 0.27	0.50 ± 0.35	240 ± 24

* The IC_{50} values (means ± SEM for 4 separate determinations) were determined in competitive binding studies using 1 nM [^{3}H]TCDD as the radioligand and different concentrations of unlabeled competitor as described in Materials and Methods; K_i values were calculated from the IC_{50} values.

† DRE binding was also determined using rat hepatic cytosol as described [26]; the results (means ± SEM for 4 separate determinations) are expressed as DRE binding observed for 50 μM concentrations of the flavones as a percentage of that observed using 5 nM TCDD in the transformation assay.

‡ ND = non-detectable.

μM 4′-chloroflavone was 3.5-fold higher than that observed for 5 nM TCDD; 50 μM 4′-bromo, 3′,4′-dichloro-, and 4′-iodoflavone all induced formation of a retarded band that was 75–240% of the intensity observed for 5 nM TCDD. The intensity of the retarded band induced by 50 μM concentrations for the remaining substituted flavones was <32% of that observed for 5 nM TCDD.

The results in Table 2 summarize the effects of 0.05, 0.5, 5.0 and 50 μM concentrations of the flavones on 5 nM TCDD-induced transformation of the rat cytosolic Ah receptor complex as determined by gel electrophoretic mobility shift assays and quantitation of the specifically bound retarded band (see Fig. 1). This study also included results for the methoxy-nitro/aminoflavones since these data were not reported previously [26]. All of the flavones significantly inhibited TCDD-induced transformation at some concentration. For example, 4′-methoxy- and 4′-iodoflavone inhibited 85 and 100% of TCDD-induced transformation at the lowest concentration (0.05 μM) used in this study. Several other substituted flavones inhibited >90% of TCDD-induced transformation at one or more concentrations, and these include flavone (5.0 μM), 4′-methoxyflavone (0.5 and 5.0 μM), 4′-aminoflavone (5.0 and 50 μM), 4′-nitroflavone (50 μM), 4′-chloro-3′-nitroflavone (5.0 μM), 3′-amino-4′-hydroxyflavone (50.0 μM), 4′-iodoflavone (0.05 and 0.5 μM, Fig. 1), 4′-methoxy-3′-nitroflavone (50 μM), 3′-methoxy-4′-nitroflavone (5.0 and 50 μM), and 4′-amino-3′-methoxyflavone (5.0 and 50 μM). There were no apparent correlations between structure-Ah receptor binding (Table 1) and structure-inhibitory (TCDD-induced transformation) activities among the substituted flavones.

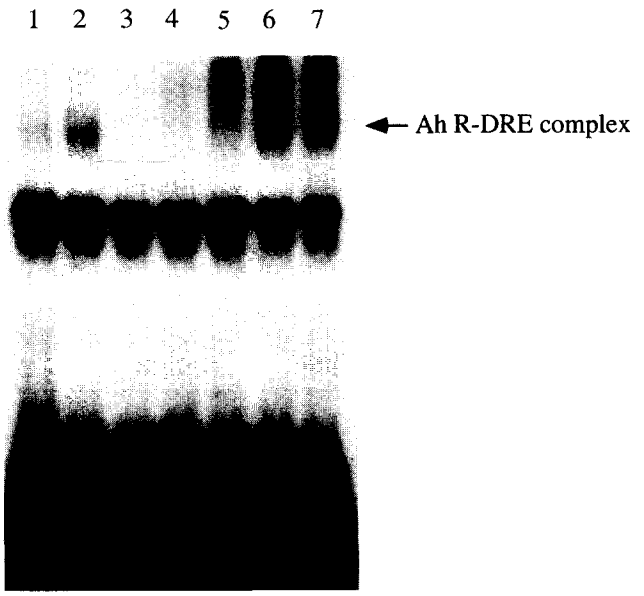

FIG. 1. Effects of TCDD, 4′-iodoflavone, and their combination on transformation of the rat heptic cytosolic Ah receptor. Rat hepatic cytosol was treated with DMSO (lane 1), 5 nM TCDD (lane 2), 5 nM TCDD plus 0.05 (lane 3), 0.5 (lane 4), 5 (lane 5), or 50 μM (lane 6) 4′-iodoflavone, and 50 μM 4′-iodoflavone alone (lane 7), and then incubated with [^{32}P]DRE; levels of the transformed DRE-Ah receptor complex were determined by gel electromobility shift assays as described in Materials and Methods. The results for the substituted flavones are summarized in Tables 1 and 2. The autoradiogram illustrated in this figure is the result of a single experiment using 4′-iodoflavone. The intensity of the specifically bound Ah receptor-DRE complex (see arrow) was non-detectable after incubation with a 200-fold excess of unlabeled DRE.

The Ah receptor agonist and antagonist activities of the substituted flavones were determined in Ah-responsive MCF-7 human breast cancer cells. At flavone concentrations from 0.01 to 10 μM, induction of EROD activity varied from 0 to 44% of the response observed for 1 nM TCDD (Fig. 2). In MCF-7 cells cotreated with 1 nM TCDD plus 0.01, 0.1, 1.0 or 10 μM concentrations of the substituted flavones, there was a concentration-dependent decrease in TCDD-induced EROD activity as illustrated in Fig. 2. All of the compounds caused a >90% decrease in induced EROD activity at concentrations varying from 0.1 to 10 μM, and the IC_{50} values for this inhibitory response are summarized in Table 3.

Previous studies have demonstrated that flavones and substituted flavones inactivate P4501A1-dependent activities by competitively interacting with the substrate binding site [26, 35–37], and therefore the effects of the substituted flavones on CYP1A1-dependent EROD activity were determined. Hepatic microsomes from Long-Evans rats treated with 5 μg/kg TCDD were incubated for 10 min with a 1 μM concentration of the substituted flavones in the presence or absence of NADH/NADPH. EROD activity was then determined, and the results are summarized in Fig. 3. All of the compounds inhibited EROD activity in the presence or absence of the reduced nucleotide cofactors, and the pattern of inhibition for the flavones was comparable, although there were significant structure-dependent differences in potency of their inhibitory effects. These data were similar to those reported previously for the 3′- and 4′-methoxy-amino/nitroflavones [26].

The Ah receptor agonist and antagonist activities of the substituted flavones were also investigated in MCF-7 cells by determining the induction of CYP1A1 mRNA levels by 1 nM TCDD, 1 μM substituted flavone, and 1 nM TCDD plus 1 μM substituted flavone. Relative CYP1A1 mRNA levels were compared with levels observed after treatment with 1 nM TCDD alone. The results (Table 3) demonstrate that 1 μM concentrations of 4′-amino-, 4′-bromo-, 4′-chloro-, 4′-iodo-, and 3′,4′-dichloroflavone induced CYP1A1 mRNA levels between 28 and 112% of that observed for 1 nM TCDD. CYP1A1 mRNA levels induced by the remaining compounds were <8% of that observed for 1.0 nM TCDD. In cells cotreated with 1 nM TCDD plus a 1 μM concentration of the substituted flavones, there was no significant decrease in CYP1A1 mRNA levels compared with cells treated with 1 nM TCDD alone. With the exception of the haloflavones, the remaining compounds were relatively weak inducers of CYP1A1 mRNA, and results of cotreatment studies suggest that these congeners

TABLE 2. Effects of substituted flavones on TCDD-induced transformation of the rat hepatic cytosolic Ah receptor

Compound		% of TCDD-induced transformation*			
		Flavones (μM)			
R (3′)	R (4′)	0.05	0.5	5.0	50
H	H	90 ± 14	24 ± 6.4†	ND†‡	27 ± 5.3†
H	OCH_3	15 ± 11†	ND†‡	ND†‡	37 ± 13†
H	NH_2	96 ± 25	48 ± 9.4†	ND†‡	ND†‡
H	Cl	58 ± 21†	39 ± 15†	137 ± 27	338 ± 92
H	Br	43 ± 6.4†	67 ± 6.4†	141 ± 17	91 ± 18
H	NO_2	83 ± 13	97 ± 18	37 ± 13†	ND†‡
NO_2	Cl	99 ± 15	106 ± 21	19 ± 8†	10 ± 6†
NH_2	OH	99 ± 16	87 ± 4	43 ± 9†	ND†‡
Cl	Cl	57 ± 8†	31 ± 7.4†	69 ± 5.3†	81 ± 12
H	I	ND†‡	ND†‡	105 ± 25	240 ± 108
NO_2	OCH_3	112 ± 10	86 ± 15	15 ± 5†	ND†‡
NH_2	OCH_3	79 ± 8.6†	29 ± 9.4†	14.4 ± 9.9†	73 ± 19
OCH_3	NO_2	58 ± 8.0†	ND†‡	ND†‡	ND†‡
OCH_3	NH_2	99 ± 14	50 ± 15†	ND†‡	ND†‡

* The effects of 5 nM TCDD plus 0.05, 0.5, 5.0 or 50 μM concentrations of the substituted flavones on transformation of rat hepatic cytosolic Ah receptor were determined by gel electrophoretic mobility shift assays as described in Materials and Methods. The intensities of the specifically bound retarded bands were normalized to that observed for 5 nM TCDD alone (100%). Results are expressed as means ± SEM for at least 4 determinations.

† Significantly lower ($P < 0.05$) than observed for 5 nM TCDD alone.

‡ ND = non-detectable.

were not Ah receptor antagonists. Of the remaining haloflavones, 4′-iodoflavone was the most effective inhibitor of TCDD-induced transformation at low concentrations (Fig. 1 and Table 2), and therefore the effects of 4′-iodoflavone (0.01 to 1.0 μM) on induction of CYP1A1 mRNA were investigated further. The results (Fig. 4) show that 0.01 and 0.1 μM 4′-iodoflavone significantly inhibited induction of CYP1A1 mRNA by 1 nM TCDD in MCF-7 cells.

DISCUSSION

Several different structural classes of compounds bind to the cytosolic Ah receptor, and these include TCDD and related polyhalogenated aromatics, polynuclear aromatic hydrocarbons (PAHs), indole-3-carbinol and related hetero-PAHs, phenanthrolines, phenanthridinones, benzocoumarins and various substituted flavonoids [23, 38–45]. Systematic structure–activity relationships have been reported for the halogenated aromatics [2, 20, 21], and there was a rank order correlation between their cytosolic Ah receptor binding affinities and their potencies as Ah receptor agonists for *CYP1A1* induction and other Ah receptor-mediated biochemical and toxic responses. Moreover, similar structure-dependent responses have been reported for other steps associated with the Ah receptor signalling pathway, and these include ligand-induced transformation of the cytosolic Ah receptor and formation of the nuclear Ah receptor complex [15, 22]. There are only limited studies on structure-dependent responses associated with ligand-induced activation of the Ah receptor for other structural classes of compounds that bind to the Ah receptor. Lu and coworkers [26] recently reported that 3′,4′-substituted methoxy-amino/nitro flavones exhibited structure-dependent Ah receptor agonist and antagonist activity. Both 3′-methoxy-4′-nitro- and 3′-methoxy-4′-aminoflavone were Ah receptor antagonists, whereas the 4′-methoxy analogs were more active as Ah receptor agonists. This study reports a more extensive structure–activity study on several substituted flavones utilizing various assays that are associated with the Ah-responsiveness of halogenated aromatics.

The results in Table 1 show that the competitive binding affinities for the rat cytosolic Ah receptor vary from 0.79 nM for 4′-iodoflavone to 4697 nM for 4′-nitroflavone. Previous Ah receptor binding studies with various substituted polyhalogenated aromatics, such as 4′-substituted-2,3,4,5-tetrachlorobiphenyls, 2-substituted-3,7,8-trichlorodibenzo-*p*-dioxins, and 7-substituted-2,3-dichlorodibenzo-*p*-dioxins, showed that the halogen-substituted analogs (I, Br and Cl) exhibit the lowest K_D values [46–49], and, in this study, the 4′-iodo-, 4′-chloro-, 4′-bromo-, and 3′,4′-dichloroflavone also exhibited high binding affinities and K_D values that were comparable to the value reported for TCDD (K_D = 1.28 nM) (Table 1). Despite the correlations observed for the halogenated aromatics and halogenated flavones, Lu and coworkers [26] have shown previously that 3′-methoxy-4′-nitroflavone also binds with high affinity to the rat cytosolic Ah receptor (K_D = 2.27 nM); in contrast, methoxy- and nitro-substituents do not enhance binding of substituted aromatics to the Ah receptor [50]. Moreover, forsubstituted halogenated aromatics, electron-donating substituents are invariably more deactivating for Ah receptor binding than electron-withdrawing substituents

[46–49]. For example, the receptor binding IC_{50} values for 7-amino- and 7-hydroxy-2,3-dichlorodibenzo-*p*-dioxin were 2.88×10^{-5} and 4.47×10^{-6} M, respectively, whereas the IC_{50} values for the electron-withdrawing nitro-substituted analog was 4.6×10^{-7} M. In contrast, 4′-nitroflavone exhibited lower binding affinities (IC_{50} = 4697 nM) than 4′-aminoflavone or 3′-amino-4′-hydroxyflavone (362 and 738 nM, respectively). The reasons for substituent-dependent differences in Ah receptor binding affinities of the substituted flavones and halogenated aromatics are unknown.

The relative potencies of substituted flavones (50 μM) to induce transformation of rat hepatic cytosolic receptor were compared with that observed for 5 nM TCDD using a gel mobility shift assay. The results (Table 1) showed the 4′-chloro-, 4′-iodo-, 4′-bromo, and 3′,4′-dichloroflavones that

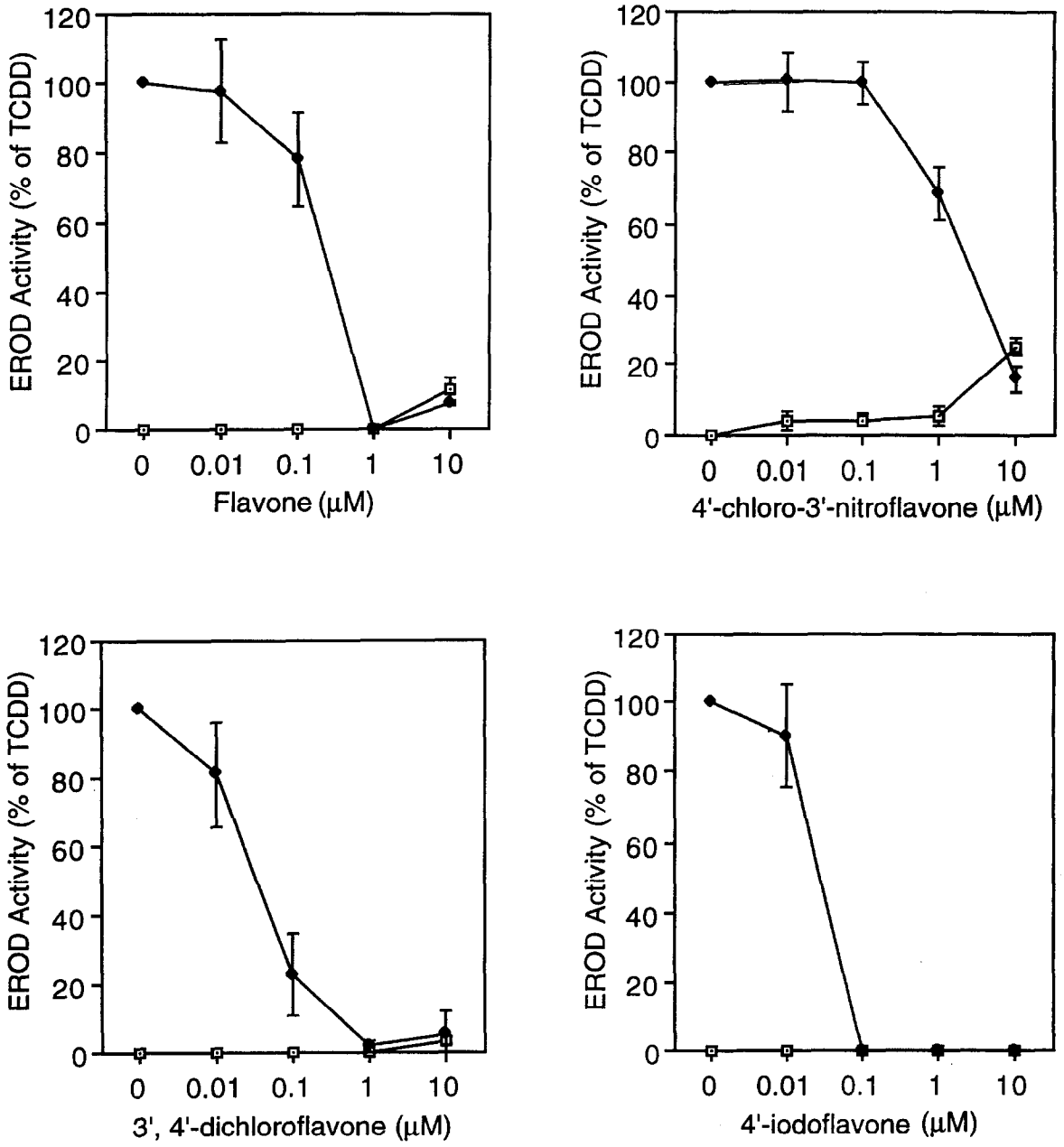

FIG. 2. Induction of EROD activity by TCDD, substituted flavones, and their combinations. MCF-7 cells were incubated for 24 hr with different concentrations of substituted flavones (□) alone or 1 NM TCDD plus different concentrations of the substituted flavones (♦), and EROD activity was determined as described in Materials and Methods. Representative data obtained for flavone (upper left), 4′-chloro-3′-nitroflavone (upper right), 3′,4′-dichloroflavone (lower left) and 4′-iodoflavone (lower right) are present. Results are expressed as means ± SD for 3 separate determinations for each data point. EROD activities in cells treated with 10 μM concentrations of the substituted flavones as a percent of the value observed for 1 nM TCDD (100%) were: 11 ± 3, 17 ± 1.6, 21 ± 2.7, 44 ± 9, 25 ± 0.3, non-detectable, 25 ± 2.4, 3.5 ± 2.9, 3.1 ± 2.7%, and non-detectable for flavone, 4′-methoxy-, 4′-amino-, 4′-chloro-, 4′-bromo-, 4′-nitro-, 4′-chloro-3′-nitro-, 3′-amino-4′-hydroxy-, 3′,4′-dichloro-, and 4′-iodoflavone, respectively.

TABLE 3. Substituted flavones: Induction of CYP1A1 mRNA levels and inhibition of TCDD-induced CYP1A1 mRNA levels and EROD activity in MCF-7 cells*

Compound		Relative % CYP1A1 mRNA		Inhibition of induced EROD activity (IC_{50}, M)
R (3′)	R (4′)	Flavone (1 μM)	Flavone (1 μM) + TCDD (1 nM)	
H	H	3.5 ± 2.9	96 ± 5.4	2.9×10^{-7}
H	OCH_3	ND†	100 ± 29	4.0×10^{-7}
H	NH_2	28 ± 9.8	98 ± 35	5.7×10^{-7}
H	Cl	70 ± 6.4	100 ± 12	1.3×10^{-7}
H	Br	32 ± 3.2	99 ± 10	9.9×10^{-8}
H	NO_2	2.8 ± 0.6	95 ± 21	2.2×10^{-6}
NO_2	Cl	4.4 ± 4.4	86 ± 29	1.8×10^{-6}
NH_2	OH	7.1 ± 4.3	83 ± 4.7	6.1×10^{-6}
Cl	Cl	75 ± 21	85 ± 47	4.7×10^{-8}
H	I	112 ± 67	141 ± 5.6	2.4×10^{-8}

* mRNA was extracted from MCF-7 cells 24 hr after treatment with 1 nM TCDD or 1 nM TCDD + the flavones (1 μM). mRNA was analyzed by northern blot analysis as described in Materials and Methods. CYP1A1 mRNA levels were normalized to β-tubulin mRNA, and relative percent mRNA levels were compared with that observed for 1 nM TCDD (100%). The results are expressed as means ± SD for at least 3 determinations for each data point. The IC_{50} values for this inhibitory response were determined by log-logit plots of the percent of maximal induced responses observed in cells cotreated with 1 nM TCDD plus different concentrations of the flavones. Maximal (100%) EROD activity was obtained with 1 nM TCDD alone (110 pmol/min/mg).

† ND = not detectable.

exhibited high competitive binding for the Ah receptor binding (IC_{50} = 0.79 to 2.28 nM) induced the highest levels of transformed cytosolic Ah receptor complex, which were 75–351% of that observed for 5 nM TCDD. All of these compounds exhibited receptor binding IC_{50} values comparable to that of TCDD, and with the exception of 4′-chloroflavone, their activity to induce transformation of the cytosolic Ah receptor was > 5000-fold lower than TCDD. The remaining flavones exhibited lower binding affinities for the Ah receptor (Table 1) and induced relatively low levels of transformed receptor (Table 2). However, results of previous studies [26] showed that 2 substituted flavones, namely 3′-methoxy-4′-nitro- and 4′-amino-3′-methoxyflavone, which bound competitively with high affinity to the Ah receptor (IC_{50} = 2.27 and 86.1 nM, respectively), induced minimal transformation (<6%) of the cytosolic Ah receptor. Thus, there was no correlation between receptor binding affinities and activity to induce transformation for these substituted flavones.

The structure-dependent Ah receptor agonist activities of the substituted flavones were investigated by determining induction of CYP1A1 mRNA levels and EROD activity in MCF-7 cells (Table 3). A 10 μM concentration of the substituted flavones induced 0–44% of EROD activity observed for 1 nM TCDD (Fig. 2), and there was no correlation between structure-binding and structure-induction activities. One micromolar concentrations of 4′-amino-, 4′-chloro-, 4′-bromo-, 3′,4′-dichloro-, and 4′-iodoflavone induced mRNA levels that were 28–112% of levels observed for 1 nM TCDD. Moreover, results of previous studies showed that the 3′-amino-4′-methoxyflavone also induces CYP1A1 mRNA levels [26]. With the exception of 4′-aminoflavone, the most active inducers of CYP1A1 mRNA levels were those congeners that bound with high affinity to the Ah receptor and induced transformation of the cytosolic Ah receptor complex. In contrast, 4′-aminoflavone exhibited relatively low Ah receptor binding affinity and did not induce receptor transformation. 3′-Amino-4′-methoxyflavone bound with higher affinity to the Ah receptor (IC_{50} = 19 nM) but also did not induce receptor transformation (Table 2), showing that for these flavones there was no correlation between their activities to induce transformation and *CYP1A1* gene expression. This is in contrast to previous studies wtih TCDD and related halogenated aromatics.

Lu and coworkers [26] previously reported that 3′-methoxy-4′-nitroflavone exhibited unusual properties since this compound exhibited high binding affinity for the Ah receptor (IC_{50} = 2.27 nM) but did not induce receptor transformation or CYP1A1 mRNA levels in MCF-7 cells. However, in MCF-7 cells cotreated with 1 nM TCDD plus 1 μM 3′-methoxy-4′-nitroflavone, there was a significant inhibition of EROD activity and CYP1A1 mRNA levels induced by TCDD. Therefore, the second phase of this study investigated the potential Ah receptor antagonist activity of the substituted flavones by determining their effects on TCDD-induced transformation of the cytosolic Ah receptor and on induction of EROD activity and CYP1A1 mRNA levels in MCF-7 cells.

Previous studies with two different structural classes of Ah receptor antagonists typified by MCDF and αNF showed that inhibition of CYP1A1 mRNA levels or EROD activity is also accompanied by inhibition of TCDD-induced transformation of the cytosolic Ah receptor [22, 33,

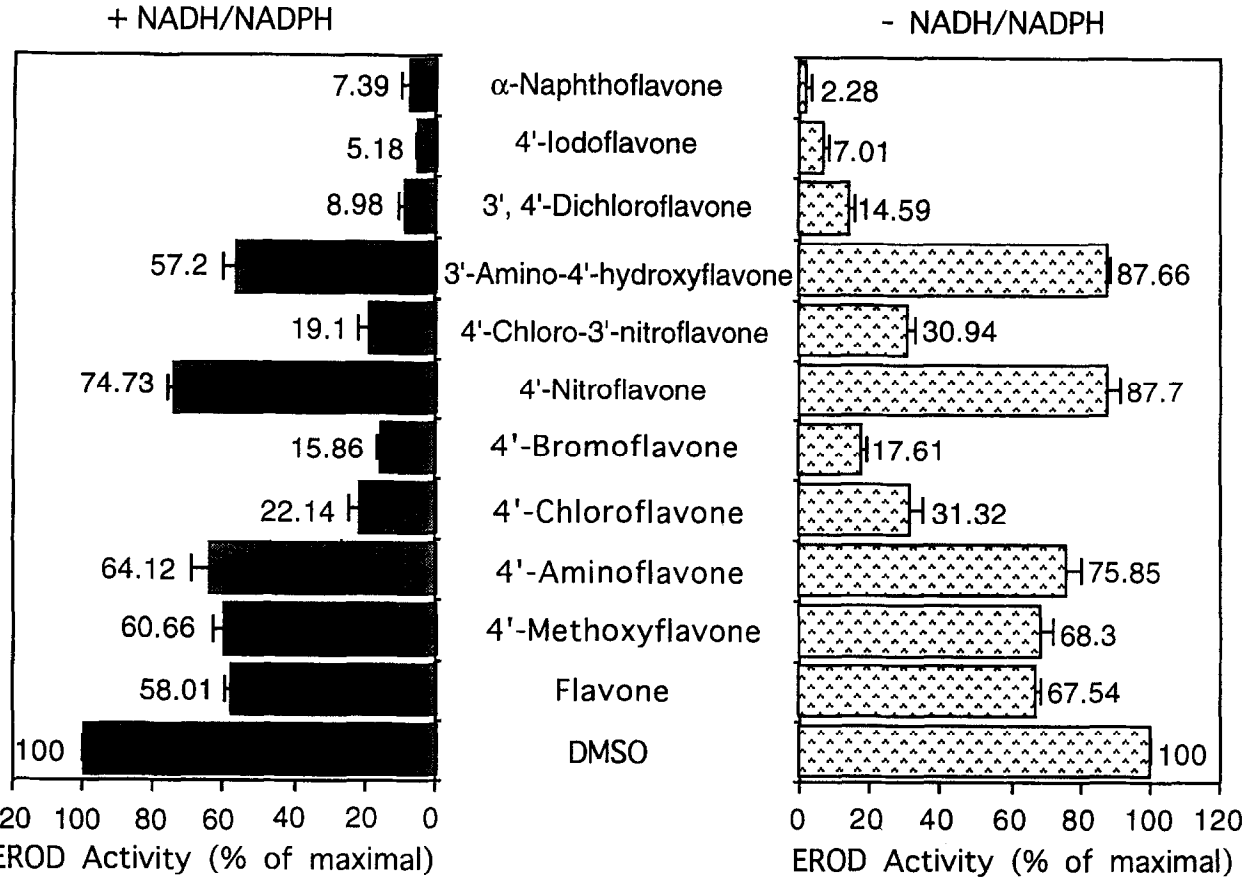

FIG. 3. *In vitro* inhibition of EROD activity by substituted flavones. TCDD-induced rat hepatic microsomes were incubated with a 1 μM concentration of the substituted flavones for 10 min, and EROD activity was determined fluorimetrically as described in Materials and Methods. EROD activity after incubation with the flavones is expressed as a percentage of activity observed after incubation of microsomes with DMSO (solvent control). Control activity = 5824 pmol/min/mg protein. The incubations were carried out in the presence (left) or absence (right) of NADH/NADPH cofactors. The results are expressed as means ± SD for 3 separate determinations.

34, 51, 52]. The results in Table 2 show that all of the substituted flavones significantly inhibited TCDD-induced transformation and with the exception of 4'-chloroflavone, 4'-bromoflavone, 3',4'-dichloroflavone, and 3'-amino-4'-methoxyflavone, >90% of induced transformation was inhibited at some concentration (Table 2 and Fig. 1). Initial studies also showed that in cells cotreated with 0.01 to 10 μM concentrations of the substituted flavones plus 1 nM TCDD, there was a concentration-dependent decrease in induced EROD activity (Table 3 and Fig. 2). The IC_{50} values for inhibition of EROD activity in MCF-7 cells varied from 0.024 μM for 4'-iodoflavone to 6.14 μM for 3'-amino-4'-hydroxyflavone. Previous studies showed that the isomeric methoxy-nitro/aminoflavones also inhibited induced EROD activity, and this was due, in part, to *in vitro* inhibition of CYP1A1 [26]. The results summarized in Fig. 3 show that after incubation of the substituted flavones (1 μM) with hepatic microsomes in the presence or absence of reduced nucleotide cofactors, there was significant inhibition of EROD activity by all the substituted flavones. These results are consistent with previous reports which show that various flavones bind to cytochrome P450 and/or inhibit P450-mediated activities [26, 35–37]. Thus, inhibition of induced EROD activity in MCF-7 cells by the substituted flavones (Fig. 3) may be due, in part, to competition with ethoxyresorufin for substrate binding sites on the enzyme.

Since previous studies showed that 4'-amino-3'-methoxy- and 3'-methoxy-4'-nitroflavone antagonize induction of both EROD activity and CYP1A1 mRNA levels induced by TCDD in MCF-7 cells [26], inhibition of induced CYP1A1 mRNA levels by the substituted flavones (1 μM) was also investigated. Only minimal inhibitory effects were observed, even though most of the compounds (with the exception of the haloflavones) exhibited relatively weak induction of CYP1A1 mRNA at the 1 μM concentration. Of the remaining haloflavones, 4'-iodoflavone was the most effective inhibitor of TCDD-induced transformation of the Ah receptor (Table 2), and interactions of 4'-iodoflavone plus TCDD showed that 0.01 and 0.1 μM concentrations of the former compound inhibited induction of CYP1A1 mRNA by 1 nM TCDD (Fig. 4). Thus, 4'-iodoflavone exhibited both Ah receptor agonist and antagonist activities.

Previous studies with halogenated aromatics and various substituted analogs showed that their activity as Ah receptor agonists was structure dependent, and that Ah receptor

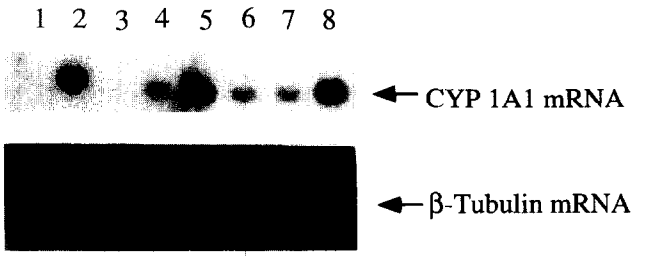

FIG. 4. Northern blot analysis of CYP1A1 mRNA levels in MCF-7 cells treated wtih TCDD, 4′-iodoflavone, and their combinations. mRNA was extracted from MCF-7 cells 24 hr after treatment with DMSO (lane 1), 1 nM TCDD (lane 2), 0.01, 0.1 and 1.0 μM 4′-iodoflavone (lanes 3 through 5, respectively), and 1 nM TCDD plus 0.01, 0.1 or 1.0 μM 4′-iodoflavone (lanes 6 through 8, respectively). mRNA levels were determined by northern blot analysis and expressed relative to β-tubulin mRNA as described in Materials and Methods. The mRNA levels in lanes 1 through 8 are expressed relative to those observed after treatment with 1 nM TCDD (100%): non-detectable, 100, 2.2 ± 2.6, 46 ± 20, 112 ± 67, 42 ± 4.9, 33 ± 11, and 141 ± 5.6 (lanes 1 through 8, respectively; means ± SD for at least 3 determinations); 0.01 and 0.1 μM 4′-iodoflavone significantly ($P < 0.05$) inhibited induction of CYP1A1 mRNA levels by TCDD.

binding affinities and ligand-induced transformation activities correlated with CYP1A1 induction potencies [2, 20]. Despite the unusually high affinity of several substituted flavones for the cytosolic Ah receptor, structure–activity correlations for these compounds were inconsistent. These data suggest that various structural classes of Ah receptor ligands induce different conformational changes in the cytosolic Ah receptor complex which differentially influence individual steps in the Ah receptor-mediated signal transduction pathway. Moreover, the results for the substituted flavones suggest that within this structural class of compounds, various substituent groups can affect markedly the activity of each individual congener as an Ah receptor agonist or antagonist. These substituent-dependent differences in the activity of the flavones may be related to ligand-induced conformational changes in the Ah receptor complex and also provide support for the proposed existence of more than one form of the Ah receptor [26, 53–55]. The existence of multiple conformations or forms of the Ah receptor complex is speculative, and future studies will utilize radiolabeled synthetic flavones that can be used to investigate both the direct binding and photoaffinity labeling of the Ah receptor.

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