A New Class Of Inducers And Inhibitors Of *CYP1A1*-Dependent Responses: 6-Substituted-3,4-Benzocoumarins

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Abstract

Using a new route of synthesis, a series of 6-substituted-3,4-benzocoumarins were investigated as inducers and inhibitors of *CYP1A1*-dependent activity. Several compounds, including 3,4-benzocoumarin, the 6-iodo- and 6-bromo-3,4-benzocoumarins, competitively bound to the rat cytosolic Ah receptor (IC_{50} =3.0 x 10^{-6}, 3.2 x 10^{-7} and 4.4 x 10^{-7} M, respectively) and induced ethoxyresorufin *O*-deethylase (EROD) activity in rat hepatoma H-4-II E cells in culture. The *in vitro* competitive receptor binding IC_{50} values for 6-phenyl- and 6-*t*-butyl-3,4-benzocoumarin were 7.0 x 10^{-6} and 1.3 x 10^{-6} M, respectively; however, neither congener significantly induced EROD activity in rat hepatoma H-4-II-E cells at concentrations as high as 10 μM. In cotreatment studies, it was shown that both 6-phenyl- and 6-*t*-butyl-3,4-benzocoumarin caused a concentration-dependent decrease in the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin(TCDD)-induced EROD activity in H-4-II-E cells. However both 6-phenyl and 6-*t-butyl*-3,4-benzocoumarin did not inhibit formation of [^{3}H]TCDD nuclear Ah receptor complexes and their DRE binding in the H-4-II-E cell line. Both compounds did not inhibit TCDD-induced *CYP1A1* mRNA levels. Thus, the 6-substituted-3,4-benzocoumarins represent a new class of ligands for the Ah receptor. The mechanism for the inhibitory effects of 6-phenyl- and 6-*t*-butyl-3,4-benzocoumarin are currently being investigated.

Introduction

The aryl hydrocarbon (Ah) receptor has been identified in organs/tissues of several animal species and in mammalian cells in culture[1]. This receptor is a ligand-binding protein that forms a nuclear receptor complex which acts as a transcriptional enhancer for specific target genes such as *CYP1A1* and glutathione-S-transferase *Ya*[2-4]. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related halogenated aromatic hydrocarbons have been extensively used to investigate the role of the Ah receptor in the induction of *CYP1A1* gene expression and other toxic and biochemical responses elicited by these compounds[1-3,5-8]. For TCDD and related congeners, there is a correlation between the levels of nuclear Ah receptor and the magnitude of the induction *CYP1A1* in mice and rat hepatoma H-4-II-E cells in culture[9-11]. This study reports the synthesis of several new 6-substituted-3,4-benzocoumarins and investigates their affinities as ligands for the rat

hepatic cytosolic Ah receptor, their concentration-dependent induction of ethoxyresorufin *O*-deethylase (EROD) activity in rat hepatoma H-4-II-E cells, and their activity as partial antagonists of TCDD-induced *CYPIA1* gene expression in this cell line.

Experimental Procedures

Chemicals All of the substituted benzocoumarins and 2,3,7,8-tetradibenzo-*p*-dioxin were synthesized in this laboratory.

Preparation of Hepatic Cytosol Rat hepatic cytosol from male Long-Evans rats was prepared according to the described procedures[1] and stored in liquid nitrogen until used.

Hydroxylapatite (HAP)assay The IC_{50} values for competitive receptor binding affinities were determined using rat hepatic cytosol (2 mg protein/ml) and HAP procedure essentially as described[12]. Different concentrations of 6-substituted-3,4-benzocoumarins were used to determine displacement curves; the IC_{50} values were determined from the plot of the percentage of [^{3}H]TCDD bound versus log concentrations of the ligands.

EROD induction in rat hepatoma cells Rat hepatoma H4IIE cells were grown as continuous cell lines in a-MEM(supplemented with 2.2 mg/ml $NaHCO_3$, 5% FCS, and 10 mM antibiotic-antimycotic solution(Sigma)). For enzyme assays, TCDD and the 6-substituted-3,4-benzocoumarins dissolved in DMSO were added to the culture flasks so that the final concentration of DMSO in the medium was 1.1%. In the cotreatment studies, the chemicals were added at the desired time. Cells were harvested and assayed for ethoxyresorufin *O*-deethylase (EROD) activity 24 hr after initial treatment determined by the method of Pohl and Fouts as described[13].

Preparation of Nuclear Extracts for Sucrose Gradient Analysis and the Gel Retardation Assay: The nuclear extracts for sucrose gradient analysis and the DRE gel retardation assay were prepared as described[14].

Sucrose Density Gradient Analysis Aliquots (300 µg) of the nuclear extracts for sucrose density gradients were loaded onto linear (5~25%) sucrose gradient and the analysis was carried out as described[14].

Gel Retardation Assay [14] Ten µg of the nuclear extracts were used for the DRE gel retardation assay. The data is presented as percent of the 1 nM TCDD-induced response.

cDNA Sources and RNA Analysis The *CYPIA1* cDNA probe and b-tubulin cDNA were from ATCC. The 1.2 kb fragment of *CYPIA1* was used to detect *CYPIA1* mRNA and the 1.3 fragment of β-tubulin was used to detect β-tubulin mRNA. The cells used for RNA analysis were treated as described for the EROD assay. RNA from the treated cells was isolated, electrophoresed, transferred to cellulose membrane, and probed as previously described[14]. The mRNA bands were quantitated on a Betagen Betascope 603 blot analyzer imaging system. The *CYPIA1* mRNA signal was standardized against the β-tubulin signal.

CYPIA1 protein detection CYPIA1 product was detected using rat cytochrome P450IA1 ECL Western blotting kit(Amersham).

Results

The 6-substituted-3,4-benzocoumarins competitively displaced [^{3}H]TCDD from the cytosolic Ah receptor. Table 1 shows that their order of binding affinity was: I- > CF_3- >

Br- > Cl- > C_3H_7- > C_2H_5- ,C_4H_9-, CH_3-, F > NO_2-, $C_{10}H_8$- > H-, NH_2- > C_6H_5-. Most of the 6-substituted-3,4-benzocoumarins induced formation of a nuclear Ah receptor complex as determined by binding to [^{32}P]DRE in a gel shift assay. Their order of DRE binding was: $C_{10}H_8$- > I-,CF3-> C_6H_5-, NH_2- > H- > Br-, C_3H_7-(iso) > NO_2-, Cl-, F-, C_2H_5- > C_4H_9-,(*t*), >CH_3-.

Table 1. 6-Substituted-3,4-benzocoumarins: Ah Receptor Competitive Binding Affinities(IC_{50}) , EROD inducibility and DRE Binding of Nuclear Extracts

Congeners X=substituent	IC_{50} (Binding) (Mean ± SD mM)	Induced DRE Binding (10 μM)[a]	EROD Inducibility (10 μM)[a]
H-	3.04 ± 0.74	27.0 ± 2.03	85.1 ± 5.38
CH_3-	1.33 ± 0.14	2.48 ± 4.29	2.86 ± 1.18
C_2H_5-	1.32 ± 0.28	14.7 ± 5.14	39.4 ± 0.96
C_3H_7-(iso)	0.65 ± 0.41	21.1 ± 2.47	37.1 ± 1.11
C_4H_9-(*t*-)	1.32 ± 0.23	7.29 ± 4.89	0
C_6H_5-	7.05 ± 1.28	33.4 ± 3.88	2.24 ± 0.13
$C_{10}H_8$-	2.18 ± 0.70	47.6 ± 4.82	24.8 ± 1.46
CF_3-	0.38 ± 0.08	37.1 ± 13.0	108 ± 3.79
NH_2-	3.84 ± 0.34	32.8 ± 6.91	30.9 ± 1.04
NO_2-	2.25 ± 2.16	18.4 ± 2.31	59.5 ± 1.46
F-	1.76 ± 0.51	13.2 ± 0.97	10.1 ± 0.40
Cl-	0.54 ± 0.15	17.0 ± 1.79	17.7 ± 6.09
Br-	0.44 ± 0.09	23.0 ± 4.65	95.1 ± 5.50
I-	0.32 ± 0.15	38.6 ± 3.65	72.42 ± 6.28

a: determined as a percentage of the response observed for 1 nM TCDD

Table 2. 6-Substituted-3,4-benzocoumarins:Inhibition of TCDD-Induced EROD Activity

Congeners (x=substituent)	% of TCDD-Induced EROD Activity[a]
H-	83.9
CH_3-	102
C_2H_5-	112
C_3H_7-(iso)	78.3
C_4H_9-(t)	35.6
C_6H_5-	35.1
$C_{10}H_8$-	89.9
CF_3-	117
NH_2-	86.6
NO_2-	174
F-	67.3
Cl-	84.8
Br--	85.5
I-	59.4

a: the cells were cotreated with 1 nM TCDD and 10 μM of the antagonists.

CH_3-, C_2H_5-, CF_3- and NO_2-substituted-3,4-benzocoumarins did not inhibit TCDD-induced EROD activity. However, the other compounds significantly decreased TCDD-induced EROD activity and their activity as inhibitors followed the order: C_4H_9-, C_6H_5- > I- > F- > C_3H_7-(iso) > H-, NH_2-, $C_{10}H_8$-, Cl-, Br-. 6-Phenyl- and 6-*t*butyl-3,4-

benzocoumarin are the most active compounds as inhibitors of TCDD-induced EROD activity.(Table 2) 6-*t*-Butyl-benzocoumarin was chosen to study the mechanism of the inhibitory effect.

Table 3 The Effects of 6-*t*-Butyl-3,4-Benzocoumarin on TCDD (1 nM)-Induced Responses

Treatment	Nuclear [^{3}H]-TCDD-Ah-R Complex (fmol/mg protein)	*CYP 1A1* mRNA (Units)	*CYP 1A1* Level (% of 5 μg β-naphthoflavone-induced rat liver microsomes)
DMSO		0.8 ± 0.9	0
TCDD (1 nM)	48 ± 5.3	24 ± 6.4	83 ± 2.7
6-*t*-butyl-BC (1 μM)	-	0.5 ± 0.6	-
6-*t*-butyl-BC (1 μM)+TCDD(1 nM)	104 ± 8.6	25 ± 0.4	-
6-*t*-butyl-BC (10 μM)	-	3.6 ± 1.8	0
6-*t*-butyl-BC (10 μM)+TCDD (1 nM)	77.3 ± 8.7	27 ± 5.6	66.4 ± 4.6*

* Significantly different from the value in TCDD group (P<0.05)

The results in Table 3 indicate that 6-t-butyl-3,4-benzocoumarin did not inhibit TCDD(1 nM)-induced nuclear [^{3}H]TCDD complex formation and *CYP 1A1* mRNA level. The compound inhibited less than 20% of TCDD (1 nM)-induced *CYP 1A1* protein level. Addition of 6-*t*-butyl-3,4-benzocoumarin to rat H4II E cells from 5 min to 1 hour prior to harvesting the cells did not result in the inhibition of TCDD(1 nM, 24 hr treatment)-induced EROD activity(data not shown) and the results are consistent with a post-translational inhibitory process. (Supported by the National Institute of Health ES03843.)

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