IDENTIFICATION AND CHARACTERIZATION OF NOVEL FLAVONE AGONISTS OF THE Ah RECEPTOR

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Introduction

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that mediates many of the biological and toxicological effects of a structurally diverse array of hydrophobic natural and synthetic chemicals[1]. Exposure to AhR agonists results in variety of effects, the most studied of which is induction of gene expression. Following ligand binding, the cytosolic ligand:AhR complex dissociates from its associated protein subunits, accumulates within the nucleus, and dimerizes with Ah receptor nuclear translocator (ARNT) protein, with the resulting conversion of the heteromeric complex into its high-affinity DNA binding form. The binding of the ligand:AhR:ARNT complex to its specific recognition site, the dioxin responsive element (DRE), stimulates gene transcription through an adjacent promoter. The AhR signal transduction pathway is present in a diverse range of species, tissues, and cell types[2]. To date, the majority of genes which respond to AhR ligands have been shown to utilize the AhR-DRE-dependent mechanism of action. Flavones represent one group of natural chemicals that have been shown to bind to and act as AhR agonists and antagonists[3, 4]. Here we describe the analysis of 37 novel flavones and benzoflavone(BNF) derivatives for their ability to bind and activate AhR using gel retardation analysis, GFP reporter assays, and RT-PCR.

Materials and Methods

The Enhanced Green Fluorescent Protein (EGFP)-Based Cell Bioassay. Mouse hepatoma (Hepa1c1c7) cells that have been stably transfected with a reporter plasmid containing the enhanced green fluorescent protein reporter gene (H1G1.1c3 cells) were maintained in alpha minimal essential medium (MEM) supplemented with 10% fetal bovine serum, penicillin/streptomycin (50 units/ml each), and 968 mg/L G418. These cells respond to TCDD and other AhR agonists with the induction of GFP in a time-, dose-, chemical-, and AhR-dependent manner. GFP activity was expressed as a percentage of that obtained with 1 nM TCDD after correction for the background fluorescence in the DMSO samples[5, 6].

RNA isolation and RT-PCR of AhR. Mouse hepatoma (Hepa1c1c7) cells were grown to 90% confluence and treated with DMSO or 1 mM chemicals for 4 hr. Total RNA was isolated with TRIzol Reagent (GibcoBRL) and stored at –80□ until further use. RT-PCR for CYP1A1 and the control HPRT was carried out as described in detail. The forward and reverse primers for mouse CYP1A1 mRNA produced a product of 280 bp between exon 5 and exon 7 of the mouse CYP1A1 gene[7]. mCYP1A1 FP: 5'-GCCTTCATTCTGGAGACCTTCC-3'and mCYP1A1 RP: 5'-CAATGGTCTCTCCGATGC-3'. Mouse hypoxanthine phosphoribosyl transferase (mHPRT) was used as an internal control and amplification of mHPRT produced a product of 177 bp[8]. Primer

sequences are as follows: mHPRT FP: 5'-GTAATGATCAGTCAACGGGGGAC-3' and mHPRT RP: 5'-CCAGCAAGCTTGCAACCTTAACCA-3'

Preparation of Nuclear Protein. Nuclear extracts of mouse hepatoma (Hepa1c1c7) cells which had been incubated with DMSO (1 μL/mL), BNF (1 μM), flavones (1 μM), or TCDD (1 nM) for 1 hr at 37□ were prepared as previously described[9]. Nuclear extracts were stored frozen at -80□ until use and protein concentrations determined using the Bradford assay[10].

Synthetic Oligonucleotides and Gel Retardation Analysis. A complementary pair of synthetic oligonucleotides containing the sequence 5'-GATCTGGCTCTTCTCACGCAACTCCG-3' and 5'-GATCCGGAGTTGCGTGAGAAGAGCCA-3' (corresponding to the AhR binding site of DRE3 and designated as the DRE oligonucleotide[11]) was synthesized, purified, annealed, and radiolabeled with [γ^{32}P]ATP as described[11]. Gel retardation analysis of nuclear extracts (14 μg protein) from cell lines was carried out as previously described[9]. The final salt concentration of the binding reaction was 160 mM KCl and 1.7 μg p[dI·dC] and protein-DNA complexes were visualized by autoradiography. The amount of [^{32}P]-labeled DRE present in the induced protein-DNA complex was quantified using a Molecular Dynamics phosphoImaging system.

Results and Discussion

EGFP Reporter Assay. Thirty seven flavones were analyzed using the high throughput EGFP reporter assay, of these 8 compounds were identified that significantly induced GFP expression (Table 1). EC_{50} values for EGFP induction by these compounds were between 10 nM and 8 μM, approximately 2 x 10^3 to 3 x 10^6-fold lower than that of TCDD (20 pM). Among them, R6 was the most potent on inducer of GFP expression, with an EC_{50} of 10 nM. These results indicate that these compounds are relatively potent AhR inducers in H1G1.1c3 cells.

Effect of Selected Flavones on Expression of CYP1A1 mRNA Levels. To establish that the transformed AhR generated by these compounds actively induced CYP1A1 mRNA *in vivo*, RT-PCR was carried out (Table 1). Total RNA was harvested following a 4 hr treatment of mouse hepatoma (hepa1c1c7) cells with the test compounds or DMSO and subsequently analyzed by PCR with CYP1A1 primers and HPRT primers. Our results showed that all of compounds, with exception of R7 and A7, induced expression of CYP1A1 mRNA levels between 12 and 81% of that induced by 1 nM TCDD. Among them, compound R6 was the most induction of CYP1A1 mRNA to a level 81% of that induced by TCDD. These results indicate that selected flavones are able to induce the CYP1A1 mRNA at the level of transcription.

Effect of Selected Flavones on AhR transformation and DNA binding in Hepa1c1c7 cells. Gel retardation analysis was performed to confirm that the inducing activity of these compounds is due to their ability to activate AhR (Table 1). Incubation of nuclear extracts with compounds R6, R9, R10, O3, and BNF stimulated formation of a protein-DNA complex that represents DNA-bound AhR. These results are consistent with their ability to activate the AhR signal transduction pathway.

Combinational Effects of Flavones on TCDD-induced EGFP Activity. To demonstrate the combinational effects of these compounds, H1G1.1c3 cells were treated with 1 μM of the indicated flavone in the absent or presence of 1 nM TCDD at 33□for 24 hr. Our results showed

that various flavones induced GFP expression to between 20 and 90% that of 1 nM TCDD (Table 2). Interestingly, in the case of cotreatment, all of the compounds decreased in TCDD-induced GFP expression, with the exception of compounds O3 and BNF, indicating that these compounds were relatively weak inducers of GFP expression. These results also indicate that several of these flavones may act as partial AhR antagonists.

Conclusions

We have been able to identify several novel flavones for their ability to activate/inhibit the AhR signal transduction pathway using a combination of ligand- and DNA-binding and reporter gene assay. In addition, cotreatment with flavones and TCDD revealed that several of these flavones were partial AhR antagonists. Overall, these results have demonstrated that the structural characteristics of several of these flavones are distinctly different from known flavonoids agonists of the AhR continuing the ongoing debates as to the structural determinants necessary for AhR agonist and antagonist activity.

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Table 1. EC_{50} and Relative Potencies of Selected Flavones in EGFP reporter gene assay, gel retardation, and RT-PCR.

Chemicals	EC_{50} (nM)[a]	Relative Potency[b]	Relative DRE binding (%)[c]	Relative CYP1A1 mRNA (%)[d]
TCDD	0.02	1	100 ± 29	100 ± 12
R6	10	2.0×10^{-3}	11 ± 2	81 ± 15
R10	30	6.7×10^{-4}	66 ± 6	35 ± 5
O3	90	1.8×10^{-4}	75 ± 12	49 ± 15
BNF	90	1.8×10^{-4}	127 ± 14	60 ± 4
R9	200	1.0×10^{-4}	31 ± 7	49 ± 6
A10	800	2.5×10^{-4}	ND	44 ± 7
A9	1000	2.0×10^{-5}	ND	24 ± 2
R8	1000	2.0×10^{-5}	2 ± 1	20 ± 5
A8	8000	2.5×10^{-6}	ND	12 ± 5
R7	ND[e]	ND	ND	5 ± 2
A7	ND	ND	ND	2 ± 2

Note. Relative potencies were determined from dose-response relationship studies measuring induction of EGFP at 24 hr.

[a]Concentration at which 50% of the maximum EGFP induction.

[b]Values are expressed relative to the EC_{50} for EGFP induction by TCDD.

[c]PhosphorImager quantitation of the amount of inducible AhR:DRE complex formed and expressed as a percent of TCDD.

[d]Values are expressed relative fold induction of CYP1A1 mRNA by TCDD.

[e]ND: Not Detectable.

Table 2. Combinational Effects of Flavones on TCDD-induced EGFP Activity.

Flavones	Flavone only	Flavone + TCDD
R6	53 ± 3	73 ± 6
R7	26 ± 1	63 ± 6
R8	47 ± 5	58 ± 7
R9	82 ± 7	98 ± 5
R10	84 ± 6	97 ± 4
O3	91 ± 4	107 ± 4
BNF	96 ± 4	114 ± 3
A7	NV[a]	75 ± 1
A8	20 ± 2	74 ± 3
A9	45 ± 5	62 ± 2
A10	61 ± 7	93 ± 3

H1G1.1c3 cells were incubated with 1 μM of the indicated flavone or 1 nM TCDD for 24 hr at 33□, and the EGFP activity was measured as described under Materials and Methods.Values are expressed as the mean ± SD of triplicate determinations relative to that induced by 1 nM TCDD (100%). [a]NV: Values were less than control.