SCREENING AND ANALYSIS OF NOVEL NAPHTHOFLAVONE LIGANDS FOR THE Ah RECEPTOR

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Introduction

The aryl hydrocarbon receptor (AhR) is a ligand-dependent transcription factor that regulates expression of a battery of genes in a wide range of species and tissues.[1] Exposure to 2,3,7,8-tetrachlorinedibenzo-p-dioxin (TCDD, dioxin), the prototypical and most potent halogenated aromatic hydrocarbon (HAH), and related compounds produces a diverse array of species- and tissue-specific toxic and biological effects, the majority of which are AhR dependent characteristics of the ligand.[2] Mechanistically, the inducing chemical enters the responsive cell and binds to the cytosolic AhR. Following ligand binding, the AhR is presumed to undergo a conformation change that exposes a nuclear localization sequence(s), resulting in translocation of complex into the nucleus. Release of the ligand:AhR from this complex and its subsequent dimerization with a related nuclear protein called Arnt converts the AhR into its high affinity DNA binding form. Binding of the heteromeric ligand:AhR:Arnt complex to its specific DNA recognition site, DRE, upstream of the CYP1A1 and other AhR-responsive genes stimulate transcription of these genes.[1,2] Detailed analysis of AhR ligand binding has predominantly focused on the structurally related HAHs and polycyclic aromatic hydrocarbons (PAHs). However, recent studies have demonstrated the ability of a structurally diverse range of chemicals to bind to and/or activate AhR-dependent gene expression suggesting that the AhR has promiscuous ligand binding site.[3,4] Therefore, identification and characterization of the spectrum of ligands for the AhR will provide insights into the structural specificity of AhR ligands, and biochemical and molecular mechanisms by which ligands can activate the AhR signalling pathway. In this study, we use a high-throughput reporter gene cell bioassay system containing a stably transfected AhR-responsive Enhanced Green Fluorescent Protein (EGFP) reporter gene and gel retardation analysis to screen and characterize novel flavonoid ligands for the Ah receptor.

Materials and Methods

EGFP Assay. The cell line H1G1.1c3 cells was created by the stable transfection of Hepa1c1c7 cells with the dioxin responsive reporter construct pEGFP1.1.[5] H1G1.1c3 cells were maintained in alpha minimal essential medium (MEM) supplemented with 10% fetal bovine serum, penicillin/streptomycin and G418. Cells were plated into black clear-bottomed 96-well microplates at 75,000 cells per well. After 24 hours, the media were replaced with nonselective media containing the chemical to be tested. In each plate, media only, DMSO and 1nM TCDD were used as blank, negative control and positive control, respectively. After 24 hours of incubation at 33°C, EGFP was measured on a fluostar microplate fluorometer (Molecular Dynamics) with an excitation and an emission wavelengths of 485 and 515 nm respectively. Dose response curves for all positive chemicals were carried out and EGFP activity was normalized by subtracting the negative control, DMSO treatment. The EC50 value of each chemical was calculated with the software Sigmaplot 2001 based on the Sigmoidal (Hill, 4 Parameter) Regression Model.[6]

Chemicals. A chemical library of flavonoid and flavone-derived chemicals was prepared as previously described and contained 57 different naphthoflavones (NF).[7]

Results and Discussion

The NFs have been separated into three groups based on their parent chemical structures: 5,6-NF (12 compounds), 6,7-NF (6 compounds) and 7,8-NF (39 compounds) (Figure 1). The activity of each NF derivatives to activate AhR dependent gene expression was tested in the AhR responsive H1G1.1c3 cells. In the first screen, all NFs were tested at two concentrations (1 μM and 10 μM). This resulted in identification of 23 compounds in which EGFP activity was greater than 50% of that induced by 1nM TCDD. Dose response curves for each of these NFs was carried out to determine their relative inducing potency, an example of which is shown in figure 2. The upper panel shows the dose response curves of the parent chemicals (5,6-NF, 6,7-NF and 7,8-NF) and the lower panel shows the dose response curves of ANF (7,8-NF) and some of its derivatives. Based on the dose response curves, the EC50 values of these compounds were calculated (Figure 1). The EC50 values of three parent naphthoflavones, 5,6-NF, 6,7-NF and 7,8-NF, were 57.6, 1182.6 and 2545.6 nM, respectively, while the EC50 of the other NFs varied widely. These results revealed that differential substitution significantly altered the relative potency of these compounds to induce AhR responsive reporter gene expression. One aspect was interesting in these analysis and that was that several derivatives of ANF, a classical AhR antagonist, were full agonists and in some cases were more potent than the classical PAH agonist BNF (5,6-NF) (Figure 1, 2). Interestingly, halogen substitution on the phenyl ring of ANF converted into an agonist. Additionally, some substitutions significantly reduced inducing potency and based on the structural analysis this appears to be due to a conformational change resulting in a shift of the phenyl ring so that it no longer lies in the plane of the rest of the molecule. Our subsequent studies have also demonstrated the ability of many of the NFs to stimulate AhR DNA binding in gel retardation analysis (data not shown). Subsequent studies will examine the ability of these NFs directly bind to the AhR and induce endogenous CYP1A1 gene expression. The results of these and other studies will provide additional insights into the structure-activity relationships for nonhalogenated AhR ligands.

Acknowledgements

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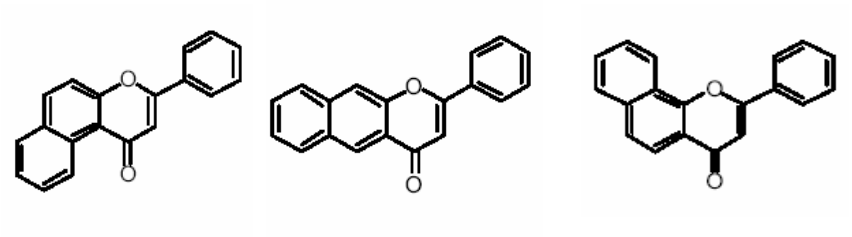

5,6-NF	6,7-NF	7,8-NF
Total: 12	6	39

ID	EC50 (nM)	ID	EC50 (nM)	ID	EC50 (nM)
BY2	57.6	BY2	1182.6	W1	2545.6
T7	26.64	BC5	>10000	AJ9	98.5
T8	563.5	BY6	>10000	V1	36.0
BY3	492.0			AJ8	574.9
AU1	170.1			U5	5.6
AT7	735.3			O3	27.9
				P5	>10000
				Q7	727.3
				AY10	2.31
				BL6	40.2
				BB9	709.6
				BL5	66.1
				AY9	27.0
				BG8	>10000

Figure 1 Structure of the three parent naphthoflavones, the total number of derivatives in the library, and the EC50 values of parent NFs and some of their derivatives to induce EGFP.

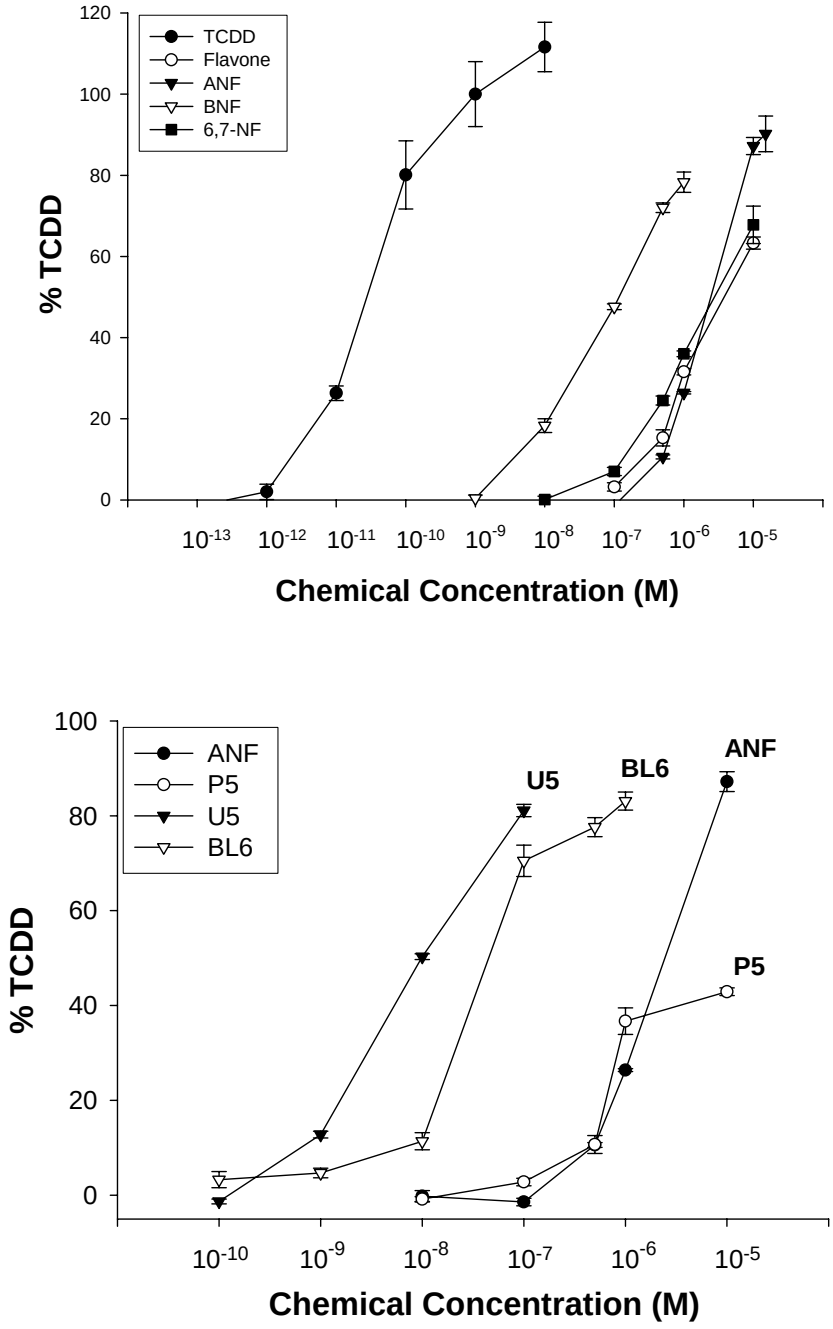

Figure 2 Dose response curves of EGFP induction by TCDD, the parent NFs (upper panel) and some of their derivatives (lower panel).