IDENTIFICATION AND ANALYSIS OF NOVEL FLAVONOID AGONISTS AND ANTAGONISTS FOR THE AH AND ESTROGEN 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 in a diverse range of species, tissues, and cell types. The most studied effect is induction of gene expression, and, the majority of AhR responsive genes, such as cytochrome P4501A1 (CYP1A1), utilize AhR dependent mechanism of action.[1] While halogenated aromatic hydrocarbons (HAHs) and polycyclic aromatic hydrocarbons (PAHs) are the prototypical ligands of the Ah receptor, it has recently identified that the AhR is activated by a structurally diverse array of hydrophobic natural and synthetic chemicals.[1,2,3,4] Given the structural diversity in AhR ligands, the physiochemical characteristics for high and low affinity ligands seems to be established.

Environmental contaminants that can disrupt the endocrine homeostasis of an organism have also gained widespread attention in recent years and numerous chemicals have been identified as having either hormone or anti-hormone properties.[5] However, like the AhR, the structural diversity and characteristics of endocrine disrupters that exert their action via nuclear receptors also seems to be depended on the estrogen receptor (ER).

The flavonoids are a diverse family of chemicals commonly found in fruits and vegetables. Members of this family exert cytostatic, apoptotic, anti-inflammatory and anti-angiogenic activities.[6,7] In addition, several flavonoids are potent modulators of both the expression and activities of specific cytochrome P450 genes/proteins and somel others have estrogenic and anti-estrogenic activity. Accordingly flavonoids have attracted attention as possible chemoprotective or chemotherapeutic agents.[6,7]

We have previously developed and analyzed a novel chemical library of flavonoids which contained ~200 compounds and the number of chemicals in each group of compounds is shown in Figure 1.[8] The ability of these compounds to activate and/or inhibit AhR- and ER- dependent gene

expression was examined by using our recently developed AhR- and ER- responsive cell bioassay systems.[5, 9]

Materials and Methods

Chemicals: TCDD was from Dr Steven Safe (Texas A&M University, USA). A chemical library of flavonoid and flavone-derived chemicals was prepared as previously described and contained ~200 different compounds.[8]

Enhanced green fluorescent protein (EGFP) assay for identification of AhR agonist: The cell line H1G1.1c3 cells was created by the stable transfection of mouse hepatoma (Hepa1c1c7) cells with the dioxin responsive EGFP reporter construct pGreen1.1.[9] H1G1.1c3 cells were maintained in alpha minimal essential medium (MEM) supplemented with 10% fetal bovine serum, penicillin/streptomycin and G418 (Gemini Bio-Products). Cells were plated into black clear-bottomed 96-well microplates at 75,000 cells per well and after 24 hours, the media were replaced with nonselective media (MEM supplemented with 10% fetal bovine serum, penicillin/streptomycin but without G418) 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 activity was measured on a Tecan GENios microplate fluorometer with excitation and emission wavelengths of 485 and 515 nm respectively. Dose response relationship analyses for all positive chemicals were carried out and induced EGFP activity was determined by subtracting the fluorescence of the DMSO sample from the treated/induced samples.[8]

Luciferase assay for identification of estrogen receptor agonist: The cell line BG1Luc4E_2 was created by the stable transfection of BG-1 cells with the estrogen responsive reporter construct as we have previously described.[5] For analysis, BG1Luc4E_2 were maintained in estrogen stripped media (ESM), consisting of MEM without phenol red supplemented with 5% dextran-coated charcoal treated FBS with the media being changed daily. Cells were plated in 24 well plates. At approximately 90% confluence, to measure the agonist effect, cells were incubated with compounds of interest only, while to measure the antagonist effects, cells were incubated with compounds and 1 nM 17β-estradiol together for 24 h. The media were then removed and the plates were rinsed with phosphate-buffered saline (PBS) and cells lysed with 100 µl of lysis buffer (Promega). Lysed samples were collected and the cell debris was pelleted by centrifugation. Luciferase activity in 25 µl of lysate was measured in Dynatech ML3000 microplate luminometer following the addition of 50 µl of luciferase reagent (Promega). Luciferase activity was normalized to the protein concentration of the cell lysate as detected using the fluorescamine assay with bovine serum albumin (BSA) as the protein standard as we have previously described.[5]

Results and Discussion

Screening of Ah receptor agonist: The activity of each compound in the library to activate AhR dependent gene expression was tested in the H1G1.1c3 cells. In the first screen, all compounds were tested at two concentrations (1 µM and 10 µM). This resulted in identification of ~40 compounds in which induced EGFP activity was greater than 50% of that induced by 1 nM TCDD. While some compounds were very potent at 1 µM (i.e. U5), some were essentially inactive, or

much less potent (i.e. BY3) and others (i.e. BL5) appeared to be toxic to cells at high concentration (data not shown). Dose response relationship analysis for each of the active compounds was carried out to determine their relative inducing potency. The EC_{50} value of each chemical was calculated from the dose response curve using a 4 parameter Hill Regression equation and the results shown in Table 1. The EC_{50} values for four parent compounds, flavone, α-naphthoflavone (ANF), β-naphthoflavone (BNF) and 6,7-naphthoflavone (6,7-NF) were 1100, 2545, 60 and 1100 nM, respectively. However, the induction potency of their respective derivatives varied significantly. The most interesting aspect in this analysis was that the most active AhR agonists found were derivatives of ANF, a classical AhR antagonist, some of these chemicals (such as O3, U5, V1, AY10) were significantly more potent than the classical PAH agonist BNF (Table 1), indicating that certain substitution on ANF dramatically changed its mechanism (presumably due to a change in its interaction with the AhR ligand binding pocket). Subsequent studies have demonstrated the ability of many of these compounds to stimulate AhR nuclear accumulation, DNA binding and induce CYP1A1 gene expression (data not shown).

Screening of estrogen receptor agonist: The ability of each compound in the library to activate estrogen receptor dependent gene expression was tested using the estrogen responsive-luciferase reporter gene containing BG1Luc4E_2 cells. In the first screen, all compounds were tested at one concentration (10 μM). This resulted in identification of 14 compounds in which luciferase activity was greater than 15% of the activity induced by 1 nM 17β-estradiol, while only 3 compounds induced luciferase activity greater than 50% of that induced by 1nM 17β-estradiol (Table 2). The interesting aspect was that most tested 6,7-NF derivatives were good ER agonists, while there were only two agonists found from flavone, ANF and BNF derivatives. We also identified 23 compounds as potent antagonists based on their ability to decrease estradiol-dependent induction of reporter gene expression by more than 50% (Table 2). Interestingly, most of these potent antagonists were ANF derivatives and some were flavone derivatives. None of the tested BNF and 6,7-NF derivatives exerted significant antiestrogenic activity. Subsequent studies have been carried out to analyze dose response curves for selective compounds to determine their relative inducing and/or inhibiting potency as described previously (data not shown).

Overall, we have identified a variety of novel potent flavonoid agonists and antagonists for the AhR and/or ER from our chemical library. Additional studies are currently underway examining the ability of these compounds to inhibit cell proliferation and activate gene expression. These results combined with our other studies will provide additional insights into the structure-activity relationships of flavonoid ligands with regards to ability to activate and/or inhibit the Ah receptor or estrogen receptor.

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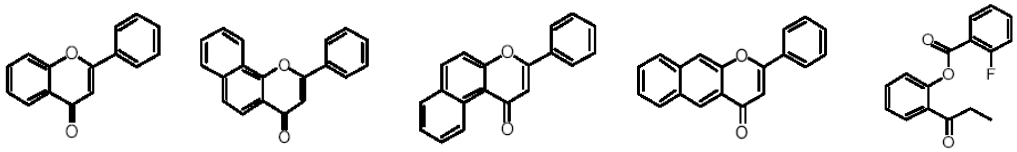

Flavone	ANF	BNF	6,7-NF	Others
37	39	12	6	100

Figure 1. Structures of parental compounds and their specific derivatives in the flavonoid library

Table 1. Relative potency (EC_{50}) of selected flavonoids to induce EGFP in H1G1.1c3 cells

Flavone		**ANF**		**BNF**		**6,7-NF**	
	EC_{50} (nM)		EC_{50} (nM)		EC_{50} (nM)		EC_{50} (nM)
Flavone	1100	ANF	2545.6	BNF	57.6	BC9	1182.6
R6	10	AY10	2.3	T7	26.6	BC5	>10000
R10	30	U5	5.6	AU1	170.1	BY6	>10000
R9	200	AY9	27	BY3	492	BY7	>10000
A10	800	O3	27.9	T8	563.5		
A9	1000	V1	36.0	AT7	735.3		
R8	1000	BL6	40.2	CA8	1180.0		
A8	8000	BL5	66.1	Z7	>10000		
		AJ9	98.5				
		AJ8	574.9				
		BB9	709.6				
		Q7	727.3				
		BG8	>10000				
		P5	>10000				
		U3	>10000				

Table 2. Estrogenic and antiestrogenic activity of flavonoids in BG1Luc4E$_2$ cells

Agonist				
Flavone	**7,8-NF (ANF)**	**5,6-NF (BNF)**	**6,7-NF**	**Other**
No 10 μM	No 10 μM	No 10 μM	No 10 μM	No 10 μM
AY9 29.4	none detected	AT7 22.3	BC5 16.0 BC6 19.4 BC7 20.5 BC8 19.6 BC9 20.0	D6 18.1 E4 25.5 E5 83.4 E6 100.7 G7 56.6 G8 29.1 AL7 45.2
Antagonist				
P2 49.7 R6 38.5 R8 43.7 R9 31.5 R10 46.6	O3 45.6 P9 40.0 Q3 28.8 Q4 30.9 Q8 39.0 R2 32.5 R5 34.3 AJ1 44.9 AJ5 46.2 BG7 38.1 BG8 35.4 BG9 31.4 BG10 32.2	none detected	none detected	H3 38.3 H4 32.3 I6 39.0 I7 35.4 I9 42.8