IDENTIFICATION OF NOVEL AGONISTS AND ANTAGONISTS OF THE Ah RECEPTOR SIGNAL TRANSDUCTION PATHWAY.

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Introduction

The aryl hydrocarbon receptor (AhR) is a ligand dependent transcription factor that is activated by the high-affinity binding of a variety of halogenated and polycyclic aromatic hydrocarbons (HAHs and PAHs, respectively)[1]. The prototypical and most potent HAH, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), results in a variety of toxic and biochemical effects, the majority of which are mediated by the AhR[2]. Expression of the toxic and biological effects are a result of ligand-dependent activation of the AhR which leads to its dimerization with AhR nuclear translocator (ARNT), nuclear accumulation, and consequent induction of gene expression. Recently, we and others have observed that the AhR can bind and be activated by a structurally diverse range of chemicals[3-5]. In an effort to better understand the structural diversity of AhR ligands and their ability to activate the AhR signaling pathway we have used a high-throughput green fluorescent protein (GFP) based recombinant cell bioassay to screen ~180 novel flavonoids and flavone-related chemicals for their ability to activate or inhibit AhR signal transduction[5,6].

Materials and Methods

Chemicals. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) was a gift from Dr. Steven Safe (Texas A&M University) and flavonoids were synthesized as previously defined[7].

AhR-Dependent GFP Reporter Gene Expression Analysis. Recombinant mouse hepatoma (H1G1.1c3) cells containing the stably transfected AhR-responsive green fluorescent protein (GFP) reporter plasmid pGreen1.1 was used. In the screening process these cells respond to AhR agonists with the induction of the GFP reporter gene activity in a time-, dose-, ligand- and AhR-dependent manner[6]. For induction studies, 75,000 cells were plated into individual wells of black clear-bottomed 96-well microplates and allowed to attach for 24 hours at 37^{o}C. After 24 hours, the media was replaced with media containing DMSO (1% maximum final concentration), TCDD or the test chemical followed by incubation for 24 hours 33^{o}C. GFP activity was measured in a Tecan microplate fluorometer (Molecular Dynamics) with excitation and emission wavelengths of 485 and 515 nm respectively. Samples were run in triplicate and wells containing media were used as blanks and subtracted as background.

Results and Discussion

The GFP bioassay has been previously established as a relatively inexpensive, quick and effective screening method for identifying compounds that exhibit dioxin like response of the AhR signaling pathway[6]. Agonist activity screens were incubated in 100 μLs of a media solution containing DMSO and the compound of interest for 24 hours at 1 μM and 10 μM concentrations, GFP response was measured in relative fluorescence units (RFU) and calculated as a percent of 1 nM TCDD response. Similarly, antagonist activity was determined by co-incubating 1 nM TCDD with the compound of interest at 1 μM and 10 μM for 24 hours. Normalization between experimental fluorescence was accomplished by adjusting the fluorometer gain so that the fluorescence level of the GFP induced by 1 nM TCDD produced relative fluorescence units of ~9000 RFU. A selection of the 10 μM induction/inhibition GFP response, presented in Figure 1, by novel compounds is expressed as a percentage of 1 nM TCDD, currently the most potent ligand of the AhR, after normalizing the response to the DMSO/media background. Initial screening of these compounds for agonist/antagonist activity has indicated that many of the compounds can bind and activate/inhibit the AhR-dependent GFP gene expression. Response of individual compounds exhibited varied induction/inhibition responses, indicated by the selected induction/inhibition data provided in Figure 1, where compounds exhibit agonist and antagonist activity, only agonist activity, or only antagonist activity.

From a total of 184 compounds agonist expression of GFP resulted at greater than 50% of 1 nM TCDD by 46 compounds at a concentration of 10μM, 9 of these compounds displayed activity equal to or greater than that of TCDD. Antagonistic activity for compounds with less than 50% 1 nM TCDD activity occurred with 58 compounds at a concentration of 10 μM, 7 of these compounds completely inhibited TCDD activity. Compounds from Figure 1 displaying both agonist and antagonist activity are AL7 and BH2. Compounds displaying mostly agonist activity, similar to β-Naphthoflavone (BNF), are W1, AY9, and BB9, and some compounds displaying mostly antagonistic activity, similar to α-Naphthoflavone (ANF), are AJ5 and AJ7.

Currently our lab is assessing the EC_{50} by fitting dose-response data to a logistic curve using Sigma Plot (SPSS, Chicago, IL), ligand and DNA binding as well as functional activity for compounds at 1 μM that display agonist activity exhibiting activity greater than 50% and antagonistic activity less than 50% than that of 1nM TCDD.

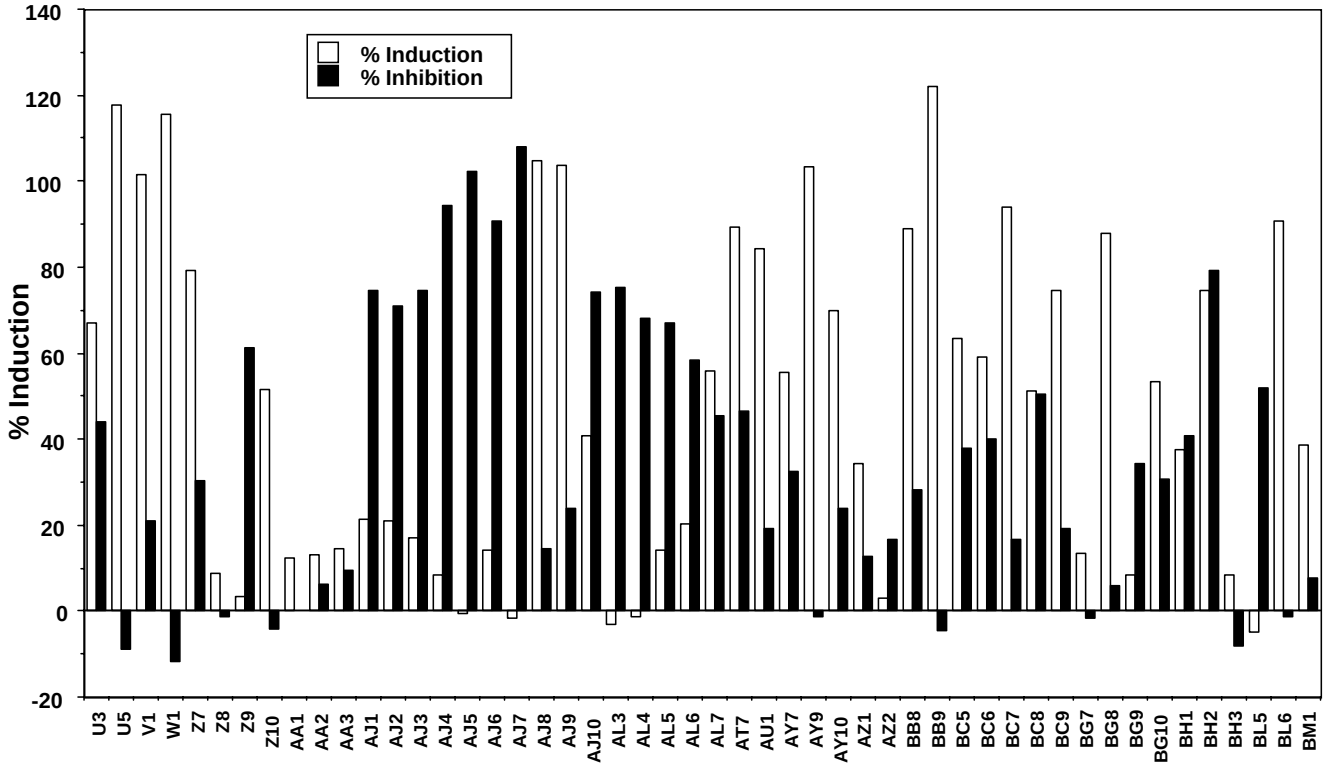

Figure 1. Selected GFP response to novel compounds as agonists and antagonists expressed as a percentage of 1 nM TCDD

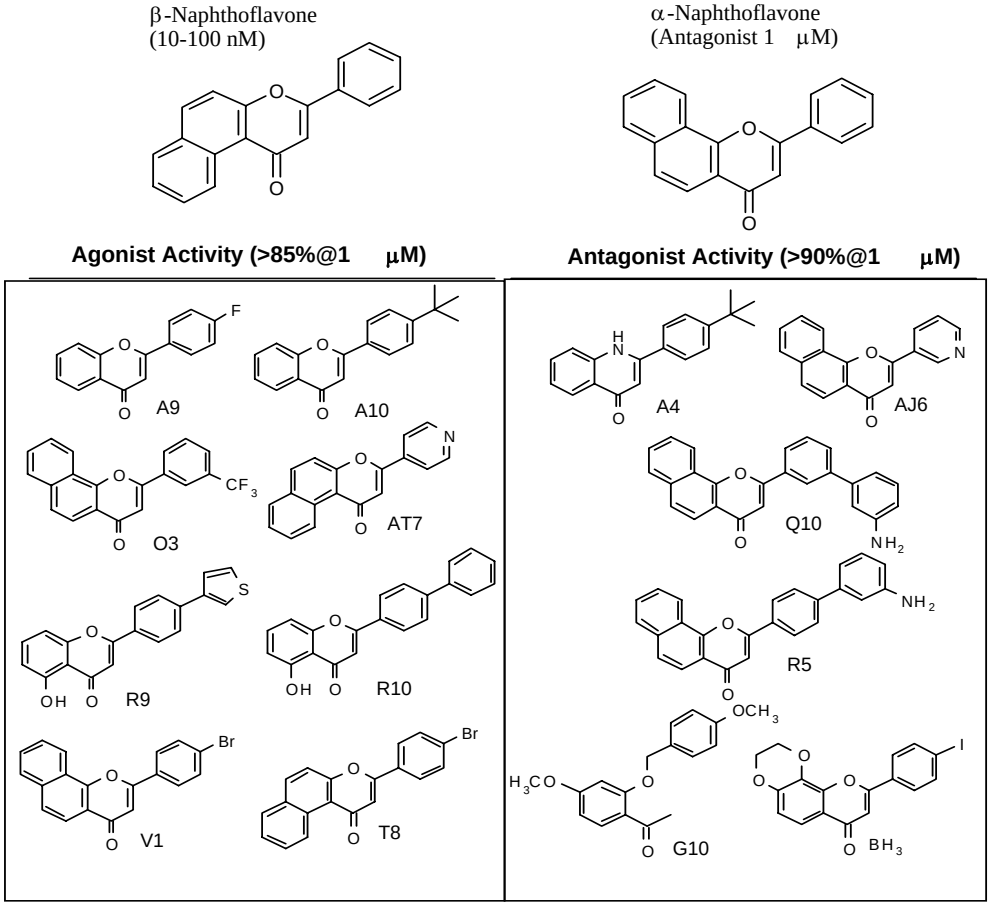

Figure 2. Selected agonist and antagonist structures of flavone and flavonoid compounds.

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